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Genetic Analysis of *C. elegans* Metabolism

by

Brendan C. Mullaney

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

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by
Brendan C. Mullaney

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Genetic Analysis of *C. elegans* Metabolism

Brendan C. Mullaney

Abstract

Regulation of nutrient consumption and storage is a complex process requiring numerous interconnected genetic pathways that monitor and respond to both environmental and internal conditions. Though we have a good framework understanding of some of the basic modules of this network, our grasp of the intricate regulation of these processes remains limited.

We have utilized the model organism *C. elegans* to investigate the genetic pathways regulating nutrient storage. To monitor nutritional status of these animals, we have utilized a number of previously described assays, and have adapted and improved others, described in chapter II. By visually monitoring aspects of nutrient storage, while simultaneously manipulating genes, either individually or in combinations, we have described a novel pathway regulating lipid storage, described in chapter III, and have begun work on a second pathway which appears to regulate the cell biology of a nutrient storage compartment, described in chapter IV.

In a forward genetic screen for genes regulating lipid storage, we isolated a mutation in *acs-3*, encoding a long chain fatty acyl-CoA synthase. These mutants exhibit elevated lipid staining intensity, and abnormally large intestinal lipid droplets. Our characterization of this gene revealed that it is expressed in a limited set of tissues, and expression of *acs-3* in the hypodermal seam cells, a stem cell-like population, is sufficient to rescue the abnormal lipid storage of mutant animals. We performed both unbiased and candidate suppressor screens, and identified a set of genes that function in the *acs-3* pathway to regulate nutrient storage. Our findings suggest a pathway in which *acs-3*, *acdh-10*, *acdh-11* and *elo-6* function to modulate fatty acyl-CoA levels, which in turn regulate *nhr-25* function, perhaps as components of *nhr-25* ligands.

The work described here sheds light on networks of genes that likely function in different tissues to regulate nutrient storage in a concerted fashion. In addition, these findings underscore the utility of genetic interrogation of metabolic networks, and demonstrate that enzymes with well-understood enzymatic activities can function in unexpected ensembles to modulated metabolism via unpredictable mechanisms.

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Chapter I: An Introduction to *C. elegans* Fat Storage and Metabolic Regulation

Abstract

C. elegans has long been used as an experimentally tractable organism for discovery of fundamental mechanisms that underlie metazoan cellular function, development, neurobiology, and behavior. *C. elegans* has more recently been exploited to study the interplay of environment and genetics on lipid storage pathways. As an experimental platform, *C. elegans* is amenable to an extensive array of forward and reverse genetic, a variety of “omics” and anatomical approaches that together allow dissection of complex physiological pathways. This is particularly relevant to the study of fat biology, as energy balance is ultimately an organismal process that involves behavior, nutrient digestion, uptake and transport, as well as a variety of cellular activities that determine the balance between lipid storage and utilization. *C. elegans* offers the opportunity to dissect these pathways and various cellular and organismal homeostatic mechanisms in the context of a genetically tractable, intact organism.

Introduction

Many core metabolic pathways found in mammals are well conserved in *C. elegans*. These include pathways for fatty acid synthesis, elongation and desaturation, mitochondrial and peroxisomal β -oxidation of fatty acids, glycolysis, gluconeogenesis and amino acid metabolism (Ashrafi, 2007; Holt and Riddle, 2003; McKay et al., 2003; O’Riordan and Burnell, 1989; Van Gilst et al., 2005a; Wang and Kim, 2003). Additionally, counterparts of a number of pathways central to regulation of mammalian metabolism appear to have similar function in *C. elegans* metabolism. Examples include fat and sugar transporters, nuclear hormone receptor and SREBP (sterol response element binding protein) transcriptional regulators, energy-sensing kinases such as AMP-

activated kinase (AMPK) and TOR kinase, as well as neuroendocrine regulators such as insulin and serotonin ((Ashrafi, 2007) and references therein).

C. elegans fat regulation differs from mammals in a number of aspects. There are no mesoderm-derived adipose cells dedicated specifically for fat storage in *C. elegans*. These animals store fat primarily in their intestinal and skin-like epidermal cells (see below). *C. elegans* also lack certain key mammalian fat regulatory mechanisms. For example, leptin, which is a core adiposity signal arising from adipose cells in mammals, has no sequence identifiable homologue in *C. elegans*. Experimental evidence, however, suggests the existence of signaling mechanisms that communicate between peripheral sites of fat storage/utilization and neural sites in homeostatic regulation of energy balance (Srinivasan et al., 2008). Other key differences include the fact that *C. elegans* are cholesterol auxotrophs, and must obtain cholesterol from their diet. Since very small dietary quantities of cholesterol are sufficient for *C. elegans* viability, it is thought cholesterol is not a required component of *C. elegans* cell membranes, but rather is necessary only for sterol-based signaling (Kurzchalia and Ward, 2003). Moreover, unlike mammals, *C. elegans* are not dependent on dietary supplies of essential fatty acids (C18:2n6 and C18:3n3) as they have the set of desaturases and elongases necessary for synthesis of these lipids (Watts and Browse, 2002). Despite the noted differences, the deep evolutionary conservation of key metabolic pathways and their regulators suggest that analyses of lipid storage pathways in *C. elegans* should be broadly informative. In support of this assertion, several newly identified *C. elegans* fat regulatory pathways have already been shown to also regulate mammalian lipid storage (McKay et al., 2003; Suh et al., 2007; Yang et al., 2006).

The field of *C. elegans* fat biology is very much in its infancy. While *C. elegans* convert excess energy into triglycerides that are stored in distinct droplet-like structures, their membrane and biophysical characteristics, associated proteins, and the extent of heterogeneity in the biochemical composition of their stored contents are largely undefined. Therefore, the aim of this review is to provide a concise overview of *C. elegans* fat storage and molecular mechanisms known to regulate the influx and efflux of lipids in these animals. Along the way, we highlight experimental approaches as well as similarities and differences in the regulation of lipid metabolism in *C. elegans* and mammals.

Tissue and subcellular characterization of *C. elegans* lipid stores.

C. elegans have a relatively simple body plan. Adult organs and tissues are comprised of an epithelial system that includes skin-like epidermal cells, a nervous system, excretory system, muscle, scavenger cells, reproductive system and sexual organs, alimentary tract consisting of the pharynx, the *C. elegans*' feeding organ, intestine and rectum. These organs are arranged within the cylindrical body shape of the animal with the alimentary tract and the gonad forming an inner tube encapsulated by an outer tube consisting of all the other tissues. These inner and outer tubes are separated from each other by a fluid-filled cavity through which secreted molecules such as insulins and lipid-laden lipoprotein-like molecules reach various tissues.

C. elegans feed on bacteria by the pumping action of the pharynx, where bacteria are physically disrupted and propelled into the lumen of the intestine. Intestinal cells are columnar polarized epithelial cells whose apical membranes face the intestinal lumen. The apical surface of intestinal cells contains microvilli necessary for absorption of

nutrients. Neighboring intestinal cells are tightly coupled by apical junctions that isolate the contents of the intestinal lumen, preventing spillover of its contents into the rest of the animal. As the only cell type with access to the intestinal lumen, these cells are also responsible for secretion of digestive lipases and peptidases (McGhee et al., 2007; Tcherepanova et al., 2000). For instance, the *C. elegans* genome encodes eight member of the α/β hydrolase lipase family. Of these eight, seven have predicted signal peptides, suggesting they may be secreted into the lumen and function as digestive enzymes. The mammalian gastric and pancreatic lipases are secreted member of the α/β hydrolase lipase family (Whitcomb and Lowe, 2007).

In addition to their role as an epithelial barrier, and in digestion and absorption, *C. elegans* intestinal cells carry out functions that are reminiscent of the drug detoxification activity of mammalian hepatocytes as well as the fat storage capacity of adipocytes. Additionally, the skin-like epidermal cells also function as a key fat storing tissue. A variety of lipid biosynthetic and degradation enzymes are expressed in intestinal and epidermal cells. For example, enzymes of the acyl-CoA synthetase, carnitine palmitoyl transferase, elongase, enoyl-CoA hydratase 3-hydroxy acyl-CoA dehydrogenase and acyl-CoA oxidase families have been shown to be expressed in the intestine, epidermis or both (Ashrafi, 2007; McGhee et al., 2007; Srinivasan et al., 2008; Van Gilst et al., 2005a). Consistent with these expression patterns, analyses of serial sections of *C. elegans* by electron microscopy (EM) reveal substantial lipid-like depots in intestinal and skin-like epidermal cells (Hall). However, identification of these depots as actual lipid-storing subcellular compartments awaits experimental verification. Biochemical fractionation of total triglyceride pools extracted from *C. elegans* has revealed that,

similar to mammals, these pools contain a range of saturated, monounsaturated, and polyunsaturated fatty acids (Ashrafi et al., 2003; Brock et al., 2007; Kniazeva et al., 2004; Tanaka et al., 1996; Van Gilst et al., 2005a; Watts and Browse, 2002). However, whether intestinal and epidermal cells have a similar composition of stored fat is not known.

Lipid staining dyes Sudan Black and Nile Red, as well as fluorescently (e.g. BODIPY) labeled fatty acids have been used to visualize stored lipids in either fixed or living animals. These dyes stain droplet-like structures primarily in intestinal and epidermal skin-like cells. Mutations or RNAi-mediated inactivation of *C. elegans* counterparts of genes that encode various lipid biosynthetic pathways or their transcriptional regulators cause substantially reduced levels of staining (Ashrafi et al., 2003; Brock et al., 2007; McKay et al., 2003; Yang et al., 2006) while loss of function mutations in a variety of other genes such as *C. elegans* counterparts of some mammalian obesity genes (Mak and Ruvkun, 2004; Mukhopadhyay et al., 2005) or a nuclear hormone receptor regulator of fat oxidation genes (Van Gilst et al., 2005a) cause increased staining.

Recently, coherent anti-Stokes Raman scattering (CARS) microscopy has been used to monitor *C. elegans* lipid stores (Hellerer et al., 2007). This methodology allows visualization of fat stores without the need for invasive techniques such as fixation or dependence on fluorescent reporters, each of which have limitations (Hellerer et al., 2007). In wild-type animals, intestinal fat stores visualized by Nile Red staining correspond well to the stores visualized by CARS microscopy. However, accumulation of epidermal fat, which can escape detection by Nile Red, is readily detectable by CARS microscopy (Hellerer et al., 2007).

Although mutagenesis and genome-wide RNAi studies have identified hundreds of genes regulating intestinal and epidermal fat depots as gauged by Nile Red fluorescent intensity or Sudan Black staining, little is known about the membrane characteristics or molecular identity of proteins that localize to *C. elegans* fat depots. A number of the best-characterized mammalian lipid droplet associated proteins have no obvious *C. elegans* homologues. These include PAT family members perilipin, ADRP TIP-47 and S3-12. By contrast, *C. elegans* has homologues of seipin (R01B10.6), leipin (H37A05.1) caveolin (T13F2.8 and C56A3.7), CGI-58 (C25A12.1 and C37H5.3), and multiple rab proteins, suggesting that some aspects of mammalian lipid droplet metabolism are likely conserved in *C. elegans*.

Hermann and colleagues have proposed that lysosome-related, terminal endocytic compartments known as gut granules, are cellular sites of *C. elegans* intestinal fat storage (Schroeder et al., 2007). These autofluorescent granules have a lipid bi-layer, are acidified and contain a vacuolar proton pump. Since these organelles do not contain LMP-1, the *C. elegans* LAMP homolog and a marker of lysosomes, they have been classified as lysosome related organelles (Clokey and Jacobson, 1986; Schroeder et al., 2007). The vital lipid dye Nile Red and BODIPY conjugated fatty acids both label these intestinal granules, and mutations that disrupt these organelles lead to a reduction or elimination of these stains (Schroeder et al., 2007). However, these mutants have relatively normal triacylglyceride content and developmental rates, suggesting that loss of this compartment does not eliminate lipid storage (Schroeder et al., 2007). Additionally, epidermal cells, which contain substantial fat depots, do not contain the autofluorescent gut granules. Thus, whether intestinal and epidermal cells rely on different subcellular

mechanisms for fat storage is not known. Similarly, whether intestinal fat is solely stored in these endocytic compartments, or whether vital dyes can only get access to these specific compartments is unresolved. One possibility suggested by Hermann and colleagues is that *C. elegans* fat storage in intestinal cells may be analogous to lamellar bodies, which are fat containing lysosome-related organelles that function in storage and release of lung surfactants (Weaver et al., 2002).

***C. elegans* lipid stores are dynamically regulated**

C. elegans fat depots are dynamic. During normal development fat droplets increase both in size and number (Hellerer et al., 2007). Interestingly, CARS microscopy volume measurements indicate that intestinal fat tends to accumulate in larger-sized droplets relative to small-sized droplets in epidermal cells. The number, size, and distribution of these droplets also change dramatically in response to environmental signals. For instance, in response to unfavorable environmental conditions, early larval stage animals enter an alternate developmental program known as the hibernating dauer state, which is characterized by accumulation of fat reserves and a subsequent shift in metabolism to favor utilization during extended periods starvation (O'Riordan and Burnell, 1989; Wadsworth and Riddle, 1989; Wang and Kim, 2003). Accordingly, hibernating dauers display a greater number and density of fat droplets visualized by staining (Kimura et al., 1997) and CARS microscopy methods (Hellerer et al., 2007). Similarly, during the adult stage, changes in diet or food deprivation elicit changes in fat composition (Brock et al., 2007; Van Gilst et al., 2005b), expression patterns of metabolic genes (Taubert et al., 2006; Van Gilst et al., 2005a; Van Gilst et al., 2005b), as well as changes in size and distribution of droplets (Ashrafi lab, unpublished

observations). While a number of gene inactivations have been identified that alter *C. elegans* fat content, the molecular mechanisms that underlie biogenesis of droplet-like fat stores and regulation of their size, number, and distribution are largely unknown.

As in mammals, the balance between *C. elegans* fat storage and fat utilization pathways is under a variety of complex transcriptional, translational, and post-translational regulatory mechanism (reviewed in (Ashrafi, 2007)). For instance, loss of function mutations as well as RNAi knockdown of *sbp-1*, the *C. elegans* homolog of mammalian SREBP, cause dramatically reduced levels of fat storage as gauged by EM, biochemical and staining methods (Ashrafi et al., 2003; McKay et al., 2003; Yang et al., 2006). A number of mammalian SREBP transcriptional targets are also regulated by *sbp-1*, including acetyl-CoA carboxylase, ATP-citrate lyase, fatty acid synthase, glycerol 3-phosphate acyltransferase and malic enzyme (McKay et al., 2003; Yang et al., 2006). In another example, *nhr-49*, a *C. elegans* nuclear hormone receptor, regulates pathways that are analogous to those under regulation of mammalian peroxisome proliferator-activated receptors (PPARs) (Van Gilst et al., 2005a). Inhibition of *nhr-49* in *C. elegans*, and PPAR family members in mice, leads to increased fat storage in these organisms, respectively (Costet et al., 1998; Van Gilst et al., 2005a; Wang et al., 2003). PPARs and *nhr-49* regulate transcription of homologous targets, including genes involved in fatty acid β -oxidation, lipid binding and fatty acid desaturation (Desvergne et al., 2006; Van Gilst et al., 2005a).

Mobilization of stored fat is ultimately dependent on flux of lipids through β -oxidation pathways. The *C. elegans* genome encodes the full complement of peroxisomal and mitochondrial β -oxidation pathway components. For many of these

components, multiple family members are encoded. Initial characterization of these genes suggests that different family members likely function under different conditions. For instance, as in mammals, increased neural serotonin signaling causes fat reduction in *C. elegans*. This is dependent on activity of a specific subset of mitochondrial and peroxisomal β -oxidation genes that are transcriptionally upregulated in response to neural serotonin signaling (Srinivasan et al., 2008). Other β -oxidation pathway components, which when inactivated cause fat accumulation, do not appear to play a role in serotonergic mobilization of fat stores (Srinivasan et al., 2008). Similarly, while both serotonin and *nhr-49* affect *C. elegans* fat, in part, through β -oxidation pathways, they do so via different β -oxidation family members (Srinivasan et al., 2008; Van Gilst et al., 2005a). It may be that the complexity and redundancy of fat oxidation pathways reflects the heterogeneity of various fat storage depots.

Flux of stored lipids through β -oxidation pathways is dependent on liberation of fatty acids from triacylglycerides through enzymatic activity of lipases. A number of well-studied mammalian lipases have close homologues in *C. elegans*, including hormone sensitive lipase (C46C11.1, for additional information see www.wormbase.org), and phospholipase A2 (C07E3.9 and C03H5.4). Desnutrin/adipose triglyceride lipase is a recently identified mammalian lipase. This enzyme contains an N-terminal patatin-like domain, and its expression is highest in adipose tissue (Villena et al., 2004). *C. elegans* has 3 homologous genes (C05D11.7, B0524.2 and D1054.1). Currently, there is little known about the physiological roles of these lipases.

Fatty acid uptake, transport and lipoproteins

Fatty acids can enter cells both through transporter independent diffusion across the cell membrane, as well as by active transport. Mammalian cells take up long chain fatty acids (LCFA) primarily via active transport (Stump et al., 2001). Fatty acid transport proteins (FATPs) as well as the protein CD36 have been demonstrated to facilitate transport of LCFAs into mammalian cells. Both families of proteins are conserved in *C. elegans*, which have two FATP family members (F28D1.9 and D1009.1), as well two CD36 homologues (Y49E10.20 and Y76A2B.6).

In yeast, bacteria and mammals, intracellular activation of free fatty acids to fatty acyl-CoAs is important for efficient uptake (reviewed in (Mashek and Coleman, 2006). Activation of fatty acids to acyl-CoAs traps these nutrients within cells, and allows them to be utilized by both catabolic and anabolic pathways. *C. elegans* have at least 12 members of the long chain acyl-CoA synthetase family, more than twice the number found in either mammals or yeast.

Fatty acid binding proteins (FABP) and acyl-CoA binding proteins (ACBP) are an additional class of proteins important in fatty acid uptake and transport. These proteins are important both for sequestration and transport of fatty acids and fatty acyl-CoAs. *C. elegans* has 9 members of the FABP family (lbp-1 to lpb-9 and EEED8.3) as well as 7 members of the ACBP family (acbp1, acbp-3 to acbp-7) as well as a membrane associated ACBP, named maa-1, involved in endosomal vesicle trafficking (Larsen et al., 2006).

C. elegans lack direct homologues to many mammalian lipoproteins, including A-I, B-48, B-100, C-II and E. Like other oviparous organisms, including some vertebrates, fish and insects, *C. elegans* use vitellogenins as protein components for intercellular

transport of lipid particles such as yolk. The *C. elegans* vitellogenin family contains 6 members (vit-1 to vit-6), some of which exhibit moderate homology to mammalian lipoprotein B-100. Yolk is produced in intestinal cells, secreted into the pseudocoelomic fluid and taken up by developing oocytes (Kimble and Sharrock, 1983). Uptake of yolk occurs in *C. elegans* in a manner similar to uptake of mammalian lipoprotein particles. The yolk receptor RME-2 is homologous to the mammalian LDL receptor, and undergoes clathrin mediated endocytosis upon yolk binding (Grant and Hirsh, 1999). Following uptake, endocytic sorting pathways are required for normal yolk trafficking within the oocyte (Grant and Hirsh, 1999).

In addition to the yolk trafficking pathway, the *C. elegans* gene *dsc-4* is a homolog of the mammalian microsomal triglyceride transfer protein. Two additional lipoprotein-receptor-like proteins are encoded in the *C. elegans* genome. *lrp-1* is homologous to the LDL receptor family member megalin, and is implicated in uptake of sterols in *C. elegans* (Yochem et al., 1999). *lrp-2* also shares strong homology to mammalian LDL receptors, but its function has not been well characterized.

Discussion

There is much to be learned about mechanisms that mediate uptake, transport, storage, and utilization of lipids. As in other organisms, *C. elegans* growth, development, reproduction, and survival is fundamentally tied to this organism's capacity to maintain adequate energy supplies in a dynamic nutritional environment. As in mammals, *C. elegans* obtain fat both from their diet and *de novo* synthesis and store neutral lipids in compartmentalized subcellular deposits. It is already clear that complex inter and intracellular mechanisms orchestrate the balance between influx and efflux of fat into and

from these compartments. However, key questions regarding the biophysical characteristics, subcellular biogenesis, and regulatory mechanisms that determine size and numbers of these deposits remain to be answered. Similarly, while lipids stored in these deposits are mobilized to fuel growth and development or meet energetic demands during periods of starvation, the molecular mechanisms that orchestrate mobilization of this stored fat are largely unknown.

Because of its incredible amenability to forward and reverse genetic analyses, the speed of identifying *C. elegans* fat regulatory genes has far outstripped the pace by which their mechanisms of function are being revealed. On-going efforts to determine the subcellular localization of protein reporter fusions corresponding to various fat regulatory genes is likely to reveal protein components of *C. elegans* fat storage compartments. Transgenic animals bearing such reporter-fusions allow for dynamic monitoring of fat droplets in the context of a variety of genetic and environmental perturbations. Although still quite limited in their scope, analyses in *C. elegans* are already revealing an astonishing complexity in the regulation of fat storage and utilization by genes whose predicted biochemical activities are seemingly redundant yet, in the context of intact organisms, have distinct functions. Finally, the amenability of *C. elegans* to repeated rounds of suppressor/enhancer screens offers the unique possibility of teasing out homeostatic mechanisms that operate across various tissues to coordinate energy balance in multicellular organisms.

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Chapter II: Methods for Analysis of *C. elegans* Fat Regulatory Pathways

Abstract

In *C. elegans* as in other animals, fat regulation reflects the outcome of behavioral, physiological and metabolic processes. The amenability of *C. elegans* to experimentation has lead to utilization of this organism for elucidating the complex homeostatic mechanisms that underlie energy balance in intact organisms. The optical advantages of *C. elegans* further offer the possibility of studying cell biological mechanisms of fat uptake, transport, storage, and utilization, perhaps in real time. Here, we discuss the rationale as well as advantages and potential pitfalls of methods used thus far to study metabolism and fat regulation, specifically triglyceride metabolism, in *C. elegans*. We provide detailed methods for visualization of fat depots in fixed animals using histochemical stains and in live animals by vital dyes. Protocols are also provided and discussed for chloroform-based extraction of total lipids from *C. elegans* homogenates that can be used to assess total triglyceride or phospholipid content by methods such as thin layer chromatography or used to obtain fatty acid profiles by methods such as gas chromatography/mass spectrometry. Additionally, protocols are provided for determination of rates of intestinal fatty acid uptake and fatty acid breakdown by β -oxidation. Finally, we discuss methods for determination of rates of *de novo* fat synthesis and Raman scattering approaches that have recently been employed to investigate *C. elegans* lipids without reliance on invasive techniques. As the *C. elegans* fat field is relatively new, we anticipate that the indicated methods will likely be improved upon and expanded as additional researchers enter this field.

Introduction

Most organisms store excess energy as fats, specifically as triglycerides along with cholesteryl esters and other neutral lipids. In addition to an energy reservoir, fat depots function as storage sites of starting material for biosynthesis of membranes and various lipid containing structures, a containment site for water-insoluble compounds such as certain xenobiotics that cannot be easily extruded from cells, and at times, as physical barriers (Garbarino and Sturley, 2009; Goodman, 2008; Olofsson et al., 2008; Schmitz and Grandl, 2009). Given the critical nature of energy balance for organismal survival, it is not surprising that its regulation in animals involves a complex interplay between multiple tissues that is manifested through behavioral, physiological, and metabolic pathways (Bournat and Brown; Kohlwein; Monda et al.; Morton et al., 2006). Although numerous cell autonomous aspects of fat regulation remain to be elucidated, the integrated and homeostatic nature of energy balance necessitates that this process be ultimately understood within the context of intact organisms. As such, *C. elegans* offers an incredible opportunity for understanding how mechanisms as diverse as behavior and metabolism are intertwined to ultimately determine an organism's fat content. Importantly, while genetic analyses in *C. elegans* have already revealed numerous fat regulatory genes, the feasibility of enhancer/suppressor genetic analyses in these animals holds the promise of unraveling homeostatic, regulatory mechanisms in ways that are not yet as easily feasible in any other animal. Moreover, the same optical advantages that long ago made Sydney Brenner select this animal for elucidating fundamental mechanisms of development, make *C. elegans* ideal for deciphering both the dynamic cell biological machines and organelles that channel nutrients and energetic resources into fat storage depots and those that utilize the contents of these depots.

Similarities and differences between *C. elegans* and mammalian fat regulatory mechanisms have been reviewed elsewhere (Jones and Ashrafi, 2009; Mullaney and Ashrafi, 2009; Watts, 2009). An important consideration is that unlike mammals, *C. elegans* do not have adipocytes dedicated to fat storage. The most prominent sites of fat accumulation in *C. elegans* are intestinal and skin-like epidermal cells. Because of its relatively large size and ease of visualization, most studies pertaining to *C. elegans* fat have, thus far, focused on depots in intestinal cells, which exhibit characteristics that are simultaneously reminiscent of those of mammalian intestinal, liver, and adipose cells. This is because the 26 intestinal cells of adult hermaphrodites function in nutrient uptake, lipid and xenobiotic metabolism, and generation and secretion of yolk, the *C. elegans* counterpart of lipoproteins/chylomicrons that are known to transport nutrients to developing embryos and likely also mediate transport of nutrients to other tissues. Given that skin-like epidermal cells do not obviously contain yolk and do not directly function in nutrient uptake, triglycerides in skin-like epidermal cells appear to be mostly in the form of storage depots. Expression of key fat biosynthetic and breakdown genes in this tissue further highlights the key, albeit somewhat overlooked, importance of skin-like epidermal cells in lipid homeostasis and storage in *C. elegans* (Greer et al., 2008; Srinivasan et al., 2008).

While the methods discussed in this chapter are primarily used to assess storage and metabolism of triglycerides, in many cases they cannot by themselves distinguish between triglycerides and other neutral lipids contained in *C. elegans*. The full identities of all neutral lipids contained in *C. elegans* are not known, but it is highly likely that they also contain cholesteryl esters and waxes.

One important limitation of using *C. elegans* for metabolic analyses is that their small size makes analyses of metabolic parameters in individual tissues a major challenge. In analogy to mammalian systems, it is quite possible that under certain conditions, one tissue may be in an oxidative, fat burning state while another tissue may be in an anabolic, fat synthesizing mode. At present, assessments of metabolic rates are best accomplished through biochemical methods. Given that these methods are applied to whole animals rather than individual tissues, they may be opaque to metabolic changes that are restricted to a specific tissue. Similarly, in addition to storage triglycerides, intestinal yolk and developing embryos within the hermaphrodite gonad contain substantial concentrations of triglycerides, therefore methods that utilize whole animal homogenates as starting points for determination of triglyceride content may fail to detect substantial changes in one tissue that are offset by changes in another tissue or compartment. For these reasons, careful attention should be paid to the selection of methods and interpretation of results.

A key consideration relevant to any method used for studying *C. elegans* fat is that metabolism in general and triglyceride content in particular are under dynamic regulation whereby the exact time within a developmental stage, diet and environmental conditions, as well as genetic background can cause dramatic effects on triglyceride content and other metabolic parameters. It is critical to minimize variables of a study such that the parameter of interest can be meaningfully investigated.

The study of *C. elegans* fat is still a new field and numerous questions regarding the basic parameters of lipid metabolism in these animals remain to be answered. At the time of writing of this chapter, there is a lack of consensus as to interpretation of certain

results obtained by various methodologies and even the proper application of some of the methodologies. While we have provided commentary based on our understanding of the methods detailed here and our personal experiences with them, we wish to emphasize that our experiences pertaining to the ease of use, sensitivity, and robustness of different methods are not necessarily shared by others also working on *C. elegans* fat regulation. Therefore, the decision as to the suitability and utilization of each method is ultimately left to the judgment of the reader.

Staining of fixed animals/histochemical methods

Histochemical stains have historically been used to visualize lipid depots in numerous species (Haltia, 2006; Kimura et al., 1997). The lysochrome most frequently used for histochemical staining of fixed *C. elegans* samples has been Sudan Black B, while Oil-Red-O (also known as Sudan Red 5B) has recently been proposed as a more suitable stain when working with fixed animals (O'Rourke et al., 2009). Both Sudan Black B and Oil-Red-O are diazo dyes with similar chemistries. In tissues, Sudan Black dyes have been used to stain neutral lipids, phospholipids, lipoproteins and lipofuscins, which are poorly characterized pigments resulting from oxidation of lipids and lipoproteins (Cerri and Sasso-Cerri, 2003; Haltia, 2006; Sun et al., 2006).

Sudan Black B and Oil-Red-O stains are minimally fluorescent. Because fluorescent readouts exhibit a larger linear dynamic range for quantitation, some researchers have advocated for the use of fluorescent, fat-soluble dyes such as Nile Red and BODIPY-labeled fatty acids on fixed *C. elegans* (Brooks et al., 2009). Additionally, LipidTox, a commercially available fluorescent dye whose chemical composition is not

publically available, is marketed by Invitrogen for visualization of fat depots in fixed samples.

To achieve relatively uniform fixation conditions and to preserve lipid conditions, mammalian tissues are typically frozen then sectioned prior to fixation and staining. Thus far, stainings of fixed *C. elegans* have been conducted on whole animals that have not been subject to sectioning. The tough *C. elegans* cuticle needs to be sufficiently permeabilized to allow for penetration of dyes without breaking the worms or stripping away lipids during the alcohol dehydration steps. Freeze/thawing of whole worms was originally used to permeabilize the cuticle. More recently, treating the cuticle with reducing agents has been suggested to yield more uniform permeabilization across staining batches than the freeze/thaw method (O'Rourke et al., 2009). The freeze/thaw method requires starting with substantially more animals since a large number are lost during the process.

Dyes and other solutions:

Sudan Black B, Oil-Red-O, or fluorescent dyes can be used for visualizing fat depots of fixed animals:

1. Sudan Black B: 16 mg/mL in 70% ethanol will give a saturated solution. This needs to be filtered (0.2 μ m filter) to remove undissolved dye.
2. Oil-Red-O stock: 0.5 g Oil-Red-O dissolved in 100 mL isopropanol. Working solution: let the stock equilibrate for 2 days, then dilute to a working solution that is 40% ddH₂O and 60% of the Oil-Red-O stock and filtered through a 0.2 μ m filter.

3. Nile Red or LipidTOX stains for fixed animals: 1 $\mu\text{g/mL}$ (3.14 μM) Nile Red in 1 x PBS; 1:1000 ratio of LipidTOX stock solution: 1 x PBS containing the fixed worms.
4. 10% Paraformaldehyde (PFA) is used to fix animals: 1 g/10 mL hot ddH₂O. Add 1-2 drops of 2 N NaOH and incubate for 15 min at 55 °C. PFA will keep for about a week at 4 °C.
5. MRWB solution is used for permeabilizing the cuticle without the use of freeze/thaw cycles: 160 mM KCL, 40 mM NaCl, 14 mM Na₂EGTA, 1 mM Spermidine HCl, 0.4 mM Spermine, 30 mM NaPIPES pH 7.4, 0.2% bME in water.

Fixation and staining of animals without freeze/thaws:

1. ~200 worms are washed into Eppendorf tubes with 1 x PBS (Phosphate Buffered Saline). Animals are allowed to settle by gravity and washed twice more, discarding supernatant. After final settling of animals, volume of suspension is reduced to 100 μL .
2. 200 μL 1 x PBS, 200 μL 10% paraformaldehyde, and 500 μL 2x MRWB are added to each sample and incubated at room temperature for 1 hour on a rotator.
3. Samples are then allowed to settle, and washed with 3 rinses of 1 x PBS. On the last wash, the volume is reduced to 100 μL . 300 μL of 1x PBS and 600 μL of 100% isopropanol are added and the sample is incubated at room temperature for 15 minutes (*see below for alternative step*).
4. Worms are allowed to settle, 900 μL of supernatant is taken from the samples, and 1 mL of prepared Oil-Red-O staining solution is added.

5. Samples are incubated overnight, pelleted using a centrifuge at 1500Xg for 30 seconds, mounted on a 2% agarose pad, and imaged.

In our experience, the following modifications to step 3 improve permeabilization of the cuticle, allowing for staining in conditions previously impervious to it: after the paraformaldehyde fixation, samples are washed 3 times with 100 mM Tris-HCl pH 7.4. After the last wash, the supernatant is aspirated down to a volume of 100 μ L. Next, 250 μ L of 40 mM DTT in 100 mM Tris-HCL and 650 μ L of 100 mM Tris-HCl are added to each sample. Tubes are incubated at room temperature for 30 minutes, animals are allowed to settle by gravity, and the DTT is washed away with 3 rinses of 1 x PBS. On the last wash, volume is reduced to 100 μ L. Next, 200 μ L of 1 x PBS and 700 μ L of 100% isopropanol are added and the samples are incubated at room temperature for 15 minutes. All other steps are as described above.

Fixation and staining of animals with freeze/thaws:

1. Several hundred worms (2-3 6 cm plates) are needed to ensure having enough animals at the end of the staining procedure.
2. Wash worms off the plate and wash away bacteria with 1 x PBS or S-Basal (1 or 2 washes).
3. After worms are washed clean of bacteria, transfer to microfuge tube in 1 mL of liquid and incubate worms on ice for ~10 min to stop pharyngeal pumping.
4. Add 50 μ L of fresh 10% Paraformaldehyde and freeze immediately in dry ice/ethanol bath (worms can be stored at -80°C at this point).

5. Freeze/thaw worms 3 times (freeze in a dry ice/ethanol bath and thaw by placing the tubes in a beaker of warm water). After the final thaw, let the animals sit on ice for 10-20 min or until fully thawed. It is critical not to thaw the tubes all the way during any freeze thaw steps. Instead, thaw for about 1 min or until the bottom 100 μ L of liquid has melted.
6. Wash fixed worms in cold 1 x PBS 3-4 times.
7. Dehydrate worms in an ethanol series consisting of 25%, 50%, 70% ethanol, keeping the worms in each for 2 minutes.
8. Aspirate off ethanol leaving 300 μ L in the tube (or transfer worms in 300 μ L to new tube).
9. Add 250 μ L of saturated Sudan Black B (in 70% ethanol) and stain animals for 4 h to overnight.
10. Spin worms down and aspirate Sudan Black B leaving $\sim$ 100 μ L.
11. Wash once in 70% ethanol to get rid of any Sudan Black B clumps which will impair picture taking and subsequent analysis.
12. Mount worms on 2% agarose pad and image.

General considerations when using histochemical stains for visualization of fat depots of fixed animals: Insufficient permeabilization of the cuticle will prevent staining while excessive permeabilization and/or poor tissue fixation can result in animals that break apart. The indicated procedures rely on either isopropanol or ethanol for sample dehydration. However, fats are readily dissolved in alcohol and thus could be easily stripped away from animals during the dehydration/staining steps. A study conducted on

mammalian cells reached the conclusion that fixation methods often alter morphology of lipid depots and produce artifacts (Fukumoto and Fujimoto, 2002). Nevertheless, these methods are routinely used for visualization of mammalian lipid depots in isolated, sectioned tissues.

While some researchers in the *C. elegans* fat field have advocated for Oil-Red-O staining as a medium-throughput method of choice with excellent reproducibility, our lab's experience is that visualization of fat depots in fixed animals suffers from extensive variability and relatively low resolution, making this approach most appropriate for comparisons between strains or conditions where the differences in fat depots are likely to be substantial. In our experience, the noted variability is inherent in the procedures of fixation and dehydration and independent of which dye is used to visualize fat depots. Moreover, in our hands, the most reproducible data is obtained when early L4 or younger animals are fixed and stained. Finally, to minimize staining variability, we label animals from one genotype (or treatment condition) with FITC and then fix and stain in the same tube as unlabeled animals from another genotype (or condition).

Biochemical methods

Biochemical methods have been used for measuring total triglyceride and phospholipid content of *C. elegans*, individual fatty acid profiles as well as estimating rates of *de novo* fatty acid synthesis and rates of fatty acid breakdown through β -oxidation.

Methods for determination of total triglyceride, phospholipid contents and their fatty acid profiles

To obtain sufficient starting material for biochemical measurements, populations of animals are grown, collected, broken apart, and total lipids are extracted. Thus far, most researchers have used generally similar methods for homogenizing populations of animals and extracting lipids. Post-extraction, however, multiple strategies have been utilized to characterize lipids and estimate triglyceride (TAG) content. These include i) fractionation of lipids by thin layer chromatography (TLC) and quantification of TAG content on the TLC plates (see below for detailed protocol), ii) TLC separation of crude lipid fractions (i.e. triglycerides vs. phospholipids), followed by methanolic saponification to convert triglycerides or phospholipids (PL) into more volatile fatty acid methyl esters that are then analyzed by gas chromatography/mass spectrometry (GC/MS) to determine the fatty acid composition (chain lengths and saturation) contained in TAG or PL fractions (Ashrafi et al., 2003), iii) separation of neutral lipids from total lipid extracts on commercially available silica-based columns, methanolic saponification of the lipid fractions, then integration of the GC/MS ion chromatogram to quantify lipid profiles and estimate total parent lipid species (O'Rourke et al., 2009; Perez and Van Gilst, 2008), and iv) use of commercially available enzymatic kits originally developed for the measurement of serum triglycerides for quantitating total TAG content (Schulz et al., 2007).

General considerations pertaining to biochemical measurements of total triglycerides extracted from C. elegans: Two general issues are noteworthy. First, regardless of measurement strategy (TLC, GC/MS, enzymatic assays), biochemical methods rely on total triglycerides extracted from populations of whole worms. As such, they do not distinguish between TAGs contained in different tissues or compartments

such as yolk. Additionally, since hermaphrodites in their reproductive period contain a substantial number of developing embryos, each of which contain a substantial amount of triglycerides (O'Rourke et al., 2009), one cannot distinguish between TAGs derived from embryos and those from non-embryonic cells if lipid extractions are performed on gravid animals. Second, given that populations of animals are used for total lipid extractions, it is necessary to normalize TAG measurements to an independent measure of the amount of material extracted. Thus far, TAG levels have been normalized to either total protein levels or phospholipid levels. The advantage of normalizing TAG levels to extractable phospholipid is that both species are isolated together during the multistep extraction procedure that minimizes random error due to sample loss during the extraction steps. The disadvantage of this normalization condition is that both TAG and phospholipids are made of similar building blocks derived from a common fatty acid pool in *C. elegans*. Many conditions (or genotypes) may affect one or both of these species. This suggests that unless phospholipids can be shown to be invariant, the TAG:phospholipid ratio should not be assumed to be a measure of total worm triglyceride levels. Alternatively, one can attempt to normalize lipid measurements to measurements of total extractable protein. While it is also possible for total protein levels to vary with condition, the chief disadvantage to using total protein as a normalization condition is that extractions of protein and lipid are performed on separate aliquots of nematodes which introduces more potential for random variability in measurements. Regardless of the normalization method utilized, multiple (i.e. > 4) independent experiments per condition are required to obtain accurate measurements as well as the reasonable assessments of the variance associated with each measurement.

Chloroform-based extraction of total lipids from populations of C. elegans

1. ~10,000 worms are used for each condition or genotype. The worm's lipid profile is dynamic. Therefore, it is critical to carefully match the populations for accurate comparisons.
2. Gently wash worms off growth plates with S-basal buffer containing 0.01% PEG into 15 mL polypropylene tubes. Use a glass pipette and avoid collecting chunks of agar along with the worms. The 0.01% PEG detergent is added to reduce loss of worms that normally stick to plastic. PEG has no influence on the TLC at the indicated concentration.
3. Pellet animals by centrifugation at 2000 X g for 30 s in a swinging bucket rotor in a table-top centrifuge at room temperature.
4. Wash pelleted worms three times by filling the tube with S-basal containing 0.01% PEG. Aspirate as much of the supernatant as possible between washes to reduce bacteria to negligible amounts. Perform one more wash with double distilled water (ddH₂O) + PEG 0.01% and reduce remaining supernatant to 1 mL.
5. Transfer 500 µL of pelleted, clean worms to a 1.5 mL polypropylene tube. Don't allow the worms to settle. Transfer 450 µL of the worm suspension to a 15 mL clean glass tubes. The 50 µL left in the 1.5 mL polypropylene tube is used for protein determination and should be frozen until the time of processing. This step is not necessary if phospholipids are to be used as a normalization parameter.
6. In sequence, add the following to the glass tube that contains the worm samples: 750 µL ddH₂O, 1.5 mL chloroform and 3 mL methanol (1:2:0.8 –

chloroform/methanol/water). Vortex and let the animals homogenize in the extractant mixture while rotating for 20 minutes at room temperature. Afterwards, add 1.5 mL chloroform and 1.5 mL 0.2 N HCl, vortex and let rotate for another 20 minutes at room temperature. Lipid extraction is based on the solubility of lipids in organic solvents and was described previously by Bligh and Dyer (Bligh and Dyer, 1959). Neutral lipids (triglycerides, waxes, cholesteryl esters) are extracted from worms with chloroform, while membrane lipids are readily extracted by the more polar organic solvent methanol. The extractant is a mixture of chloroform, methanol and water, initially in volume proportions of 1:2:0.8, which are miscible, producing a single phase. The total volume of this mixture can be adjusted as needed. After worms are homogenized in this mixture, more water and methanol are added and the mixture separates into two phases. The lipids remain in the bottom (chloroform) layer, while the more polar molecules like proteins and sugars partition into the top (methanol-water) layer. Proportions of components of the extractant mixture can be adjusted for more effective extraction of certain lipid species. The protocol described here is optimized for recovery of triglycerides.

7. During the second incubation period prepare the “blank top phase” tube that will be used for cleaning the lower (chloroform) phase of the extracted lipids. In a clean glass tube add 1.2 mL ddH₂O, 1.5 mL 0.2 N HCl, 3 mL chloroform and 3 mL methanol. Vortex and separate the phases by centrifugation at 1000 X g for 5 minutes in a swinging bucket rotor in a table-top centrifuge at room temperature. Remove the bottom phase using a clean glass pasteur pipette.

8. Separate the extractant mixture in the extraction tube into two phases by centrifugation at 1000 X g for 5 minutes in a swinging bucket rotor in a table-top centrifuge at room temperature. At this point, a layer formed by worm debris at the interface of bottom and top phases can be seen. Using a pasteur pipette, carefully transfer the bottom/chloroform phase from the extraction tube to the “blank top phase” tube. Transfer all of bottom phase and do not worry if some of top phase comes along.
9. Vortex and separate the mixture into two phases by centrifugation at 1000 X g for 5 minutes in a swinging bucket rotor in a table-top centrifuge at room temperature. Using a pasteur pipette, discard as much as possible of the top phase.
10. To ensure loading of similar amounts of each sample, it is necessary to recover lipid extractions in the same volume. The bottom phase is dried under vacuum at 45 °C. Higher temperatures are not advised without a stream of an inert gas, because of the risk of oxidation of lipid species. Pre-heat a water bath to 45 °C. Fit the tube containing the bottom phase and introduce the vacuum source half-way from the sample to clear the solvent fumes. Let the bottom phase dry. Then resuspend the dried sample in 400 µL 1:1 chloroform/methanol solution, vortex for 10 s and carefully transfer to a 1.5-2.0 mL clean glass tube using a pasteur pipette. Store at 4 °C.
11. To determine the loading amounts, protein concentration of each sample is measured and used to normalize volumes. To measure protein concentrations, thaw previously frozen 50 µL sample (from step 5 above) and add 200 µL of urea protein extraction buffer (7 M urea / 2 M thiourea / 4% CHAPS / 50 mM HEPES pH 7.4 / 1

mM EDTA / 5 mM TCEP, Tris(2-carboxyethyl)phosphine). The buffer can be pre-made without the reducing agent TCEP, which should be added fresh. Vortex and incubate the solution rotating at 37 °C for 1 h. Clear the sample by centrifuging at 10,000 X g for 5 minutes in a table-top centrifuge at room temperature. Use 25 µL to measure the protein concentration through the Bradford assay. Calculate the volume necessary to load 1.5 µg of protein.

Several methods have been published for extraction of *C. elegans* lipids (Perez and Van Gilst, 2008; Schulz et al., 2007; Soukas et al., 2009; Watts and Browse, 2002; Zhang et al., 2010). Although each protocol is slightly different from the method detailed above, they all rely on a chloroform-based organic solvent system to achieve lipid extraction. The significant differences are largely in terms of which developmental stage, growth temperature, and number of animals that are used as starting points. Additionally, fractionation of lipid species through solid phase chromatography has been accomplished with an extraction procedure that uses chloroform, methanol and water in different proportions than that the one described here (Perez and Van Gilst, 2008).

Visualization and quantitation of extracted lipids by TLC

We have chosen to provide a detailed methodology for visualization and quantitation of different lipid species by TLC as its set-up is least dependent on specialized equipment. The reader is referred to published methods for use of GC/MS (Perez and Van Gilst, 2008; Soukas et al., 2009; Watts and Browse, 2002) and enzymatic methods (Schulz et al., 2007).

Thin-layer chromatography (TLC) is a straightforward chromatographic procedure that has been used to analyze lipids extracted from *C. elegans* (Zhang et al., 2010). TLC is commonly developed using a glass plate coated with silica as absorbent. A sample of extracted lipids is loaded near one edge of the plate, which is then dipped into a chamber containing a mixture of solvents. The mixture of solvents rises by capillary action and carries the lipids on through the plate. Polar lipids bind tightly to the polar silica while more hydrophobic species tend to interact poorly with the absorbent and move faster on the plate. Spraying the plate with appropriated dyes allows the detection of fractionated lipids. Lipid species can be compared to standards for identification and quantification purposes. This section describes a TLC based method optimized for quantification of triglycerides extracted from *C. elegans* and based on normalization to protein levels.

1. Prepare one pre-coated silica gel 60 plate for sample loading. Use gloves any time you handle the TLC plate. With a pencil, draw a straight line 1.5 cm from one edge of the plate. Mark spots for sample loading along this line, 1 cm apart between samples and 3 cm from the sides of the plate. In a 20 x 10 cm plate this allows enough space to run up to 12 samples including several standards. Draw a line for the solvent front 1 cm from the top of the plate.
2. The TLC chamber is prepared 30 minutes prior to use by filling it with 100 mL of the chosen solvent system (this volume may vary depending on the size of the chamber) to a depth of no more than 5 mm from bottom of the chamber. The liquid phase for fractionation of triglycerides consists of hexane, diethyl ether and acetic

acid with volume proportions of 7:3:0.1. The acetic acid enables separation of free fatty acids. Mix the solvents in a beaker and pour into the chamber. Open the chamber only if necessary so as to maintain the atmosphere saturated with solvent vapors.

3. Using regular pipette tips, load normalized volumes of each sample onto the plate in duplicate. Load four spots with triglyceride standards to generate a standard curve from 1 to 6 μg . Each plate should contain its own standards. Carefully place the TLC plate into the chamber and check if the baseline is above the level of the solvent. Keep the chamber covered while the liquid phase develops on the plate. When the solvent front reaches the line drawn on the top of the plate, remove the plate from the chamber and allow it to dry in a fume hood.
4. Lipids species can be visualized through destructive, semi-destructive or nondestructive methods. Compared to the methods currently available (references), the destructive lipid-staining dye phosphomolybdic acid is most effective in revealing triglycerides in a range from 0.001 to 10 μg and suitable for quantification. However, this dye reacts with double bonds thus saturated fatty acids will be underestimated. Another option is the nondestructive fluorescent dye Primuline (Zhang et al., 2010), used at 0.05% concentration. However, Primuline has a smaller dynamic range than phosphomolybdic acid. Iodine vapors have also been described for TLC plate staining although this semi-destructive dye has the disadvantage of interacting with double bonds and the visualized signal fades quickly (Meyers and Meyers, 2008).

5. Upon selection of the dye of choice, it should be sprayed onto the TLC plate with a sprayer until an even layer is formed. Allow the excess dye to dry. Next, place the TLC plate in a pre-heated, 100 °C oven pre-heated for 10 min. Avoid heating the TLC plate too long or at higher temperatures since this can increase the background. Remove the plate from the oven and cool at room temperature. At this point, it is possible to see the lipid species as dark bands over a green background.
6. Scan the TLC plate using a minimum resolution of 300 dpi. Any software for image analysis can be used here. Using the triglyceride standards, calculate the R_f value (divide the distance of the band from the origin by the distance travelled by the solvent front) for the triglyceride region. The value should be between 0.2 and 0.8 to ensure a good separation. Next, use the standards as parameters to define the triglyceride region. Plot the grayscale profile of the region. Subtract the background using the baseline of the profile. Calculate the area for each sample. Construct a density X absolute quantity graph for the standards. The function can be used to determine the absolute quantities of each sample.

Normalization to parameters other than protein The methodology detailed above uses total protein extracts for the purposed of normalization. In this strategy, amount of lipid samples loaded onto TLC plates are adjusted to reflect similar protein concentrations. TLC plates can be run without volume correction if triglyceride:phospholipids ratios are to be reported. In doing so, it is important to recognize that similar to TAGs, there are multiple phospholipids with different mobilities.

Alternative methods for quantitation and analyses of extracted lipids: If fatty acid chain lengths and saturation levels are to be determined, the method of choice post total lipid extraction is GC/MS. TAGs and PL can be separated from each other as described above by TLC or by using silica based columns, converted to fatty acid methyl esters and then quantified by GC/MS (O'Rourke et al., 2009; Perez and Van Gilst, 2008).

A number of researchers have used data generated from GC/MS profiles to directly quantitate TAG levels (again, since the source of these TAGs are populations of animals, results are reported as ratios normalized to phospholipids, proteins, or other parameters). The following assumptions are thought to hold if this methodology is used for quantification of triglycerides and phospholipids: i) conversion of triglycerides and phospholipids to methyl esters occurs with the same efficiency in all samples, ii) levels of interfering substances that suppress ionization are constant throughout extracts, iii) carryover from injection to injection does not exist, and iii) samples are highly pure. For example, triglycerides are not contaminated with cholesterol esters or waxes, which will also yield fatty acid methyl esters upon methanolic saponification.

Finally, use of commercially available enzymatic measurement kits has been reported for TAG quantitation (Schulz et al., 2007). A consideration in using these kits is that purified neutral lipids are efficiently solubilized to solutions required for enzymatic activities on which these kits are based.

Biochemical measurements of metabolic rates

Though a number of genetic pathways have been identified that modulate lipid storage in *C. elegans*, the underlying mechanisms causing the observed lipid phenotypes

are generally not well understood. Methods have been published for determining rates of *de novo* fat synthesis and fat breakdown by β -oxidation.

de novo fat synthesis: by employing a mixed-isotope labeling approach, Perez *et al.* (2008) have demonstrated a method for determining the relative abundance of dietary, *de novo* synthesized, and maternally contributed fatty acids in a population of animals. To employ this method, animals are fed an equal mixture of ^{12}C and ^{13}C labeled bacteria. Lipids are extracted from animals fed this mixed-isotope diet, and the fatty acids are analyzed using gas chromatography/mass spectrometry. Dietary fatty acids consumed from this mixture are nearly completely comprised of ^{12}C carbons, if they were synthesized in ^{12}C labeled bacteria, or nearly completely comprised of ^{13}C carbons, if they were synthesized in ^{13}C labeled bacteria. The number of ^{12}C s and ^{13}C s in each fatty acid can be calculated based on the molecular weight of fatty acids determined through mass spectrometry. A small amount of isotope contamination is present in both heavy and light media, which is accounted for in calculations that determine the origin of fatty acids. *de novo* synthesized fatty acids incorporate a mixture of ^{12}C and ^{13}C , clearly distinguishing them from dietary fatty acids. The maternally contributed fatty acids are revealed in the increased abundance of ^{12}C relative to ^{13}C fatty acids in animals fed an equal ratio of ^{12}C and ^{13}C labeled bacteria. Because maternally contributed fatty acids are nearly completely comprised of the naturally predominant ^{12}C , this bias reveals the fraction of fatty acids from this source. A detailed protocol for this method, including calculations necessary to distinguish relative contribution of fatty acid sources based on isotope distribution, can be found in Perez *et al.* (2008).

Fat oxidation: this method utilizes tritium labeled oleic acid to determine the rate of fatty acid β -oxidation by measuring the rate of production of labeled water. L4 animals are rinsed of bacteria and exposed to oleic acid conjugated to BSA as their only exogenous energy source. After an incubation period, excess oleic acid is precipitated out of solution and bound to the charged DOWEX column, while the tritiated water is eluted, measured and related to protein content. The measurement of fatty acid oxidation is sensitive to the concentration of worms, oleic acid added to each sample, and the length of incubation.

1. Prepare the assay mix: dry 50 μ L 10 mM oleic acid and 13 μ L 5 μ Ci / μ L oleic acid [9, 10- ^{3} H(N)]. To the oleic acid mixture, add 550 μ L 1 mM NaOH, vortex, then add 450 μ L freshly made 18.33 mg/mL fatty acid free BSA. Vortex and let incubate at room temperature for 15 minutes prior to use.
2. 10,000 worms are used for each sample and 3-5 samples per condition are run for each experiment. Animals are washed off plates into 15 mL tubes using S-basal with 0.01% PEG, and rinsed 3 times to remove bacteria.
3. Pelleted worms are transferred in 480 μ L of liquid into a 2.0 mL microfuge tube. Next, sequentially transfer 20 μ L of this sample into a 1.5 mL microfuge tube, add 30 μ L H₂O, and freeze for protein determination. Separately, prepare three 2.0 mL tubes with 460 μ L of S-basal with 0.01% PEG for background.
4. Add 40 μ L assay mix to the samples and rotate at room temperature for 1 hour.
5. Prepare columns by tightly packing 0.1 g glass wool into a Pasteur pipette. Wash 15 mL DOWEX 1 x 8 200-400 Mesh column material (Invitrogen) in 50 mL H₂O.

Centrifuge material at 2880 X g for 1 min. Discard supernatant and repeat washes twice more. Add washed DOWEX to prepared pipettes until the settled material is 4 cm above the top of the glass wool. Run H₂O through the columns to prevent them from drying out.

6. After incubation of samples, add 540 μ L 10% TCA in the same sequence the assay mix was added. Centrifuge at 10,000 X g for 5 minutes.
7. Transfer 750 μ L supernatant to a new 2.0 mL microfuge tube. Add 250 μ L PBS and 100 μ L 5 M NaOH.
8. Run samples through prepared DOWEX columns into 20 mL scintillation tubes. Elute each sample with 1 mL of H₂O, add 10 mL scintillation fluid to elutant, and vortex for 30 seconds.
9. Prepare one background tube with 2 mL water and 10 mL scintillation fluid, and a specific activity tube with 4 μ L of assay mix and 10 mL scintillation fluid. Run samples through a scintillation counter, and normalize decays per minute to protein content.

Visualization of fat depots and fat metabolism using vital dyes

Vital dyes were originally developed to overcome the limitations of fixed stains such as lipid droplet morphology changes as well the ability to non-invasively image lipid stores in live cells. They have been used to visualize lipid depots in numerous species ranging from yeast to mammalian cells (Fowler and Greenspan, 1985; Teixeira et al., 2003; Wolinski and Kohlwein, 2008). There are several reasons for using vital dyes. First, they are generally quite easy to use. Second, they provide spatial resolution allowing for monitoring events within distinct cellular compartments or tissues. Third, as

in application of fat stains to fixed samples, they allow for examination of individual animals rather than measurements derived from populations. Additionally, given the fluorescent properties of commonly used vital dyes, they generally afford great sensitivity and dynamic range. Fourth, emission spectra of vital dyes such as Nile Red inform on the general composition of the environment in which the dye is resident (Greenspan and Fowler, 1985). Fifth, in *C. elegans*, the non-destructive nature of the staining facilitates genetic analysis.

The two vital dyes most commonly used to study *C. elegans* fat metabolism are Nile Red and BODIPY-labeled fatty acids. Although inactivation of numerous well established, evolutionarily conserved fat regulatory genes alter accumulation of these dyes in *C. elegans* in predictable ways, their utilization for the study of *C. elegans* fat metabolism has recently been called into question (Brooks et al., 2009; Hellerer et al., 2007; O'Rourke et al., 2009; Spanier et al., 2009). A discussion of these criticisms is beyond the scope of this chapter and the validity of the concerns raised against use of vital dyes to study *C. elegans* fat is, in our opinion, open to debate.

General considerations for use of vital dyes Nile Red and BODIPY- labeled fatty acids: Nile Red fluorescence depends on the polarity of the environment in which it is dissolved. In a polar solvent such as water, Nile Red's fluorescence intensity is very weak and red shifted relative to its properties in a hydrophobic environment, such as a lipid compartment (Greenspan and Fowler, 1985). As commonly used in *C. elegans* thus far, "low concentrations" of Nile Red fed to living animals label acidic granules of the gut (O'Rourke et al., 2009; Schroeder et al., 2007). The cell biological and physiological roles of these granules are currently poorly understood, however, changes in numerous

energy metabolic parameters and genes previously associated with fat regulation alter Nile Red staining patterns of these granules (Ashrafi et al., 2003; Jo et al., 2009; Jones et al., 2009; Menzel et al., 2007; Soukas et al., 2009; Srinivasan et al., 2008). Nile Red resident in these compartments is efficiently excited using either the 532 or 561 nm laser lines available in many fluorescent microscopes. If using a broad-spectrum source such as a xenon or mercury lamp, excitation bandpass filters that overlap with the 530-580 nm spectral region are appropriate (i.e. excitation filters for Cy3 or mCherry). The emission maxima in these intestinal compartments reside in the 620-635 nm range. Most emission filters used to capture red emissions (i.e. Cy3, mCherry) efficiently capture Nile Red emissions from this lipid storage site. Because Nile Red's fluorescence properties are very sensitive to its environment, a modest change in lipid content or composition can result in a significant change in Nile Red fluorescence (Diaz et al., 2008). As such, Nile Red has the potential to reveal changes that may be opaque to other methods (Greenspan and Fowler, 1985).

BODIPY-labeled fatty acids, which are also used as vital dyes to study *C. elegans* fat metabolism, have a number of properties that distinguish them from Nile Red. BODIPY-labeled fatty acids are hydrophobic, and therefore exhibit relatively specific staining of fat deposits. Unlike Nile Red, BODIPY fluorescence is insensitive to its environment and therefore its fluorescence intensity is proportional to dye quantity. For these reasons, BODIPY-labeled fatty acids exhibit a more compact dynamic range in reporting changes in lipid content or composition. However, at the typical concentrations used thus far, BODIPY-labeled fatty acids appear to stain all significant sites of lipid deposition. These include gut granules also stained by “low concentrations” of Nile Red,

other intestinal lipid depots, as well as lipid stores in the hypodermis, and lipid depots in oocytes and embryos. If concentrations of Nile Red are increased to those typically used for visualization of fat deposits in mammalian systems (2-10 mM), these other fat depots observable by BODIPY-labeled fatty acids also become observable by Nile Red.

Regardless of which vital dye is used, it is critical that comparisons between various strains or conditions be made in animals of the same developmental stage. This is because across developmental stages, lipid storage varies significantly in response to both developmental and reproductive energy demands.

Vital BODIPY-labeled fatty acid staining

Stock solution: dissolve BODIPY-labeled fatty acid (Invitrogen D3823, BODIPY 500/512 C1, C12) to a concentration of 1 mg/ml in DMSO for a concentration of 2.47 μ M. This solution is usually stable for extended periods of time when wrapped in foil and kept at -20°C.

1. Routine staining is conducted on 6 cm plates containing 10 mL NGM and seeded with OP50 (detailed instructions for making NGM plates and growing OP50 are available in the Maintenance of *C. elegans* chapter of www.wormbook.com).
2. For each 6 cm plate, dilute 0.2 μ L of BODIPY-labeled fatty acid stock into 500 μ L PBS and apply to the surface of the plate. When applied to the 10 mL volume of the 6 cm plate, this yields a dilution factor of 1:50,000 from the 1 mg/mL stock (49 nM). It is important that the solution cover the surface of the plate evenly.
3. Leave plates open in a cell culture hood until the surface of the plate has dried.

4. Apply 100-200 synchronized L1s to each plate (a protocol for obtaining synchronized L1 larvae is described in detail in the Maintenance of *C. elegans* chapter of www.wormbook.com).
5. Animals can be visually assayed 48 to 72 hours later using a fluorescence dissecting scope with green fluorescence filters such as those used for eGFP or fluorescein.

Vital Nile Red staining for visualization of intestinal acidic vesicles

Stock solution: dissolve Nile Red powder (Sigma N3013) in acetone at a concentration of 500 μ M.

The staining methodology is essentially the same as vital BODIPY-labeled fatty acid staining described above. For each 6 cm plate, dilute 1 μ L of Nile Red stock into 500 μ L PBS, and apply to the surface of the plate. When diluted into the 10 mL volume of the 6 cm plate, this yields a final concentration in the plate of 50 nM. It is important that the solution cover the surface of the plate evenly. Nile Red fluorescence in animals is visualized using a red fluorescence filter (i.e. Texas Red or TRITC filters).

With the relatively low concentration of Nile Red used in this protocol, staining in live animals is only apparent in the intestine. Though significant amounts of lipid are stored in the *C. elegans* hypodermis and deposited in developing oocytes and embryos, these tissues do not stain with low Nile Red concentrations (i.e. 50-200 nM). In addition, intestinal Nile Red staining is predominantly limited to acidic lysosome-related gut granules (Schroeder et al., 2007). Other intestinal lipid depots, such as yolk, do not appear to stain with low levels of Nile Red. Though low concentrations of Nile Red do

not stain all lipid depots, changes in staining intensity with this protocol have generally led to identification and elucidation of various fat regulatory pathways.

Visualizing hypodermal, intestinal and gonad lipid stores using Nile Red

Higher concentrations of Nile Red allow for visualization of lipid stores in the hypodermis and depots such as yolk in the intestine and gonad. Similar concentrations of Nile Red (2-10 mM) have typically been employed to visualize lipid depots in mammalian cells (Fowler and Greenspan, 1985). A key feature to the implementation of this method is that Nile Red exhibits blue-shifted fluorescence properties when staining these environments versus acidic granules in the intestine. Nile Red that is bound to the hypodermal storage droplets or the yolk stores in the intestine/gonad is most selectively and efficiently excited with light in the 470-540 nm range. Common laser emissions available in most confocal microscopes such as 488, 514, or 532 nm are perfectly suitable to excite Nile Red in these depots. For compound epifluorescence microscopes equipped with a mercury or xenon source lamp, excitation bandpass filters typically used to excite GFP or YFP (better) can be used to excite Nile Red in these tissues. Nile Red exhibits a large Stokes shift, with emission maxima in these tissues occurring between 560 and 585 nm. To selectively image these stores, emission filters that collect 540-580 nm light should be used. Typically, the emission filters for YFP offer good coverage of this spectral region. The red (620-640 nm emission maxima) acidic compartments can also be observed in this type of experiment using the appropriate filters, but they tend to be obscured by higher levels of background fluorescence.

Some considerations to the implementation of this method in contrast to the other vital dye methods described in this chapter are i) *levels of background staining*. Nile Red has been employed at lower concentrations (50-200 nM) in screens because of the high signal to noise ratio characteristic of this assay. Nematodes can be observed *in situ* and do not need to be washed free of excess dye in order to detect phenotypic change. ii) *variability*. At lower concentrations, Nile Red staining intensities of acidic compartments typically exhibit a standard deviation of approximately 15-20% in wild-type *C. elegans*. Using the high concentrations of Nile Red, significantly more variability among individuals in a population is observed which may reflect the combined variance of lipid stores in multiple different tissue compartments.

Stock solution: make a 10 mM Nile Red stock solution in DMSO by dissolving 10 mg of Nile Red powder in 0.314 mL of DMSO. Heat to 65 °C and vortex vigorously to dissolve.

1. For one 6 cm plate that contains 10 mL of NGM-agar medium, aliquot 5 µL of warm Nile Red stock to a fresh tube, immediately dilute with 250 µL of OP-50 suspension and mix by vigorous pipetting.
2. Transfer all of this mixture to the plate, and allow it to absorb overnight in the dark into the plate.
3. Transfer animals to be stained onto the plate. Animals can be cultured on the plates for 2 h to several days. Labeling of yellow-orange lipid stores becomes apparent after 2 h.
4. Prepare animals for imaging as described in below.

Image capture and quantitation

To capture images of Nile Red or BODIPY-labeled fatty acid fluorescence, animals must be mounted on a slide, and imaged using a compound fluorescence microscope. The process of mounting *C. elegans* is described in detail in the Methods in cell biology chapter of www.wormbook.com.

All fluorescent images should be acquired using identical settings and exposure times to allow direct comparisons. Images used for quantitation should be captured such that pixel intensities are linear with exposure time and below saturation. The most important aspect of quantitation is obtaining sufficiently high quality images. Because of the natural curvature of the *C. elegans* intestine and epidermal skin-like cells, it is virtually impossible to obtain single plane images of animals where the entire area stained by vital dyes are within focus. In our experience, vital dye fluorescence intensities of the anterior region of the worm (e.g. from head to the vulva) are typically representative of fluorescence intensities obtained from whole animals, although a number of exceptions have been noted. At least 8-10 worms from each genotype/condition should be randomly selected for imaging and quantitation. Substantially larger number of worms can be imaged and quantitated using automated microscopy settings or automatic fluorescence scanning devices if set up appropriately to identify individual worms and distinguish them from background.

Fatty acid uptake

Fatty acids conjugated to the fluorescent probe BODIPY have long been used to monitor fatty acid uptake in mammalian cell culture experiments (Schaffer and Lodish, 1994). The BODIPY fluorophore is insensitive to both the pH and polarity of its environment, making fluorescence intensity a good measure of dye concentration. BODIPY conjugated fatty acids are taken up in cells using the fatty acid uptake machinery, making them an ideal reagent for monitoring this process. As noted above, current studies of fatty acid β -oxidation can also provide an independent estimate for rates of intestinal absorption since that assay is dependent on uptake and breakdown of labeled oleate.

Stock solution: dissolve BODIPY-fatty acid (Invitrogen D3823, BODIPY 500/512 C1, C12) to a concentration of 1 mg/mL in DMSO for a concentration of 2.47 μ M.

1. To make the assay solution, spin down one volume of *E. coli* OP50, pour off the supernatant, and resuspend in one volume of S-Basal.
2. Add 1.5 μ L BODIPY stock per mL of assay solution.
3. Aliquot 500 μ L assay solution into 1.5 mL Eppendorf tubes.
4. Rinse synchronized L4 or day 1 adult animals off plates into conical tubes with S-Basal.
5. Allow animals to settle to the bottom of the tube for 5 minutes, aspirate off the S-Basal, leaving $\sim$ 1 mL in the bottom of the tube.
6. Pipette the worms from the bottom of the tube in a 30 μ L volume and add to the BODIPY assay solution.

7. Rotate the tubes for 20 minutes at room temperature. It is important that animals are allowed to take up fatty acid for a precise period of time.
8. To stop fatty acid uptake, place tubes on ice, and allow animals to settle to the bottom of the Eppendorf tube.
9. Pipet 5 μ L from the bottom of the tube onto a slide containing an agar pad chilled to 4 °C. This will immobilize the animals.
10. Capture images using a compound fluorescence microscope. It is important that the same exposure time be used for all conditions tested. Exposure time must be empirically determined to allow for sufficiently bright images without allowing any pixels to reach saturation in any condition.

Raman scattering based methods of lipid imaging in *C. elegans*.

The past ten years have witnessed the development of novel imaging techniques based on Raman scattering that enable the imaging of chemical bond-specific vibrational modes within living tissues (Wachsmann-Hogiu et al., 2009). The most widely applied technique in both *C. elegans* (Hellerer et al., 2007; Le et al., 2009; Morck et al., 2009) and other organisms, termed Coherent Anti-Stokes Raman Scattering (CARS) is a non-linear optical technique that detects light emission due to the Raman scattering of specific molecular bonds within a sample (Evans and Xie, 2008). CARS microscopy in principal can be based on any abundant molecular vibration that exhibits a relatively strong Raman emission. For imaging lipids, the C-H vibrations that occur in the region 2800-2900 cm^{-1} are commonly used since lipids are enriched in aliphatic C-H bonds. Contrast for CARS is determined by differences in the relative concentrations of C-H bonds (or any other

abundant, strong Raman scattering bond) within cells, with the CARS signal being proportional to the square of the concentration of a particular bond within a focal volume.

While this is a promising and rapidly evolving technology for non-destructive, non-invasive imaging of lipid structures, there are some caveats to interpreting CARS images of the lipid compartments in *C. elegans*. The first is that while triglycerides in lipid droplets likely represent a significant component of the CARS C-H vibrational signal, other neutral lipids such as cholesterol esters and esters of fatty acids with fatty alcohols (waxes) may also contribute significantly to this signal. It is also not clear whether CARS C-H imaging can readily distinguish triglyceride-containing lipid droplets from triglyceride-containing yolk. CARS signals are considerably weaker than fluorescence signals, making comparisons between CARS data and fluorescence-based data difficult. CARS may underestimate lipids present at somewhat lower densities due to its quadratic dependence on bond abundance combined with the presence of a large non-resonant background signal that is characteristic of this imaging technique. In addition, light scattering in thick samples, such as adult animals, may limit full sample optical sectioning. Finally, while the excellent optical sectioning capabilities of CARS can result in high resolution images, fully sampled, complete Z-series should be used for all quantifications to avoid under sampling errors derived from measuring signals that may vary with imaging plane within the nematode. Given the relatively recent extensions of this methodology to imaging *C. elegans* lipid stores, it is not clear what the best practices are for quantifying the amount of lipid underlying the CARS signal from individual nematodes in a population. As this technology is rapidly evolving, future

refinements of these techniques should improve the general applicability of this technique to *C. elegans*.

Discussion

The histochemical, vital dye, Raman scattering, and chloroform extraction methods discussed here provide independent methods for determining total fat content of animals. Substantial changes in total fat content caused by genetic alterations, pharmacological treatments, or environmental manipulations are expected to be detectable by each of these methods. However, for more modest changes, it is critical that dynamic range and sensitivity of each of the methods be carefully considered in order to select the most appropriate strategy. Additionally, investigation of molecular parameters such as gene expression patterns, behavioral parameters such as feeding and movement rates, and physiological parameters such as growth rate and rate of progeny production are often necessary to understand homeostatic mechanisms that underlie energy balance in intact organisms.

An exciting opportunity presented by *C. elegans* is the possibility to study fat metabolism as a dynamic process whereby energetic resources are partitioned among various compartments and in multiple tissues, each of which may be responsive to various cues of energy demand. As such, there is a great need for development and refinement of methods that allow for dynamic investigation of metabolic flux with spatial and temporal resolution. Combined with the amenability of *C. elegans* to rapid genetic and genomic analyses and experimental advantages of *C. elegans* for investigation of cell biological parameters, study of lipid metabolism in this nematode has great promise to further our understanding of metabolic processes in all living organisms.

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**Chapter III: Regulation of *C. elegans* Fat Uptake and Storage by Acyl-CoA
Synthase-3 is Dependent on NR5A Family Nuclear Hormone Receptor *nhr-25***

Summary

Acyl-CoA synthases are important for lipid synthesis and breakdown, generation of signaling molecules and lipid modification of proteins, highlighting the challenge of understanding metabolic pathways within intact organisms. From a *C. elegans* mutagenesis screen, we found that loss of ACS-3, a long-chain acyl-CoA synthase, causes enhanced intestinal lipid uptake, *de novo* fat synthesis, and accumulation of enlarged, neutral lipid rich intestinal depots. Here, we show that ACS-3 functions in seam cells, epidermal cells anatomically distinct from sites of fat uptake and storage, and that *acs-3* mutant phenotypes require the nuclear hormone receptor NHR-25, a key regulator of *C. elegans* molting. Our findings suggest that ACS-3 derived long chain fatty acyl-CoAs, perhaps incorporated into complex ligands such as phosphoinositides, modulate NHR-25 function, which in turn regulates an endocrine program of lipid uptake and synthesis. These results reveal a link between acyl-CoA synthase function and an NR5A family nuclear receptor in *C. elegans*.

Introduction

The ability to store nutrients, primarily in the form of triacylglycerides, is necessary to provide energy during periods when energy demands exceed caloric intake, however excessive diversion of nutrients for storage can impair growth, reduce reproductive fitness, and is associated with adverse health effects (Guh et al., 2009; Reeves et al., 2007). Numerous studies in organisms ranging from bacteria to humans have identified enzymatic components of metabolism as well as its neural and endocrine regulators. Although many of these components have already been characterized at the biochemical and structural levels, understanding the precise mechanisms by which

metabolic activities of various tissues are coordinated and how dysregulation of these processes may result in aberrant metabolic regulation remain significant challenges.

One complexity of metabolism is that many enzyme families are composed of multiple and seemingly redundant members. For instance, at least 26 known or predicted acyl-CoA synthase (ACS) genes are found in the human genome (Watkins et al., 2007). The ACS family of enzymes catalyzes conversion of free fatty acids to acyl-CoA derivatives. Although the ACS enzymes have been classified based on their substrate chain length preference into very long chain, long chain, medium and short chain families, the precise physiological roles of most of these enzymes are unknown. Another complexity of metabolism is that the same metabolic intermediates can, depending on the pathway into which they are channeled, have vastly different fates. For instance, acyl-CoAs can be substrates for fat breakdown through mitochondrial or peroxisomal β -oxidation, or alternatively they may be substrates for synthesis pathways, generating triglycerides for storage or structural components of membranes such as phospholipids (Coleman et al., 2002). Additionally, acyl-CoAs serve as substrates for lipid modification of proteins and function as signaling molecules either on their own or as components of more complex molecules, such as phosphoinositols and sphingolipids (Bartke and Hannun, 2009; Black and DiRusso, 2007; Faergeman and Knudsen, 1997; Payraastre et al., 2001). Thus, the consequences of misregulation of an individual ACS are quite difficult to predict even when the biochemical activity of that ACS is known.

C. elegans is an emerging model for the study of metabolism in the context of intact animals (Jones and Ashrafi, 2009; Mullaney and Ashrafi, 2009; Watts, 2009). Like most free-living organisms, *C. elegans* normally live in environments where food

availability is dynamic. Thus, they are able to sense nutrient levels and coordinate their behavioral, physiological, and metabolic responses accordingly. Despite obvious differences, many known mammalian fat regulatory mechanisms are well conserved in *C. elegans*. These include fat and sugar uptake, transport, breakdown, and synthesis pathways, transcriptional regulators such as nuclear hormone receptors (NHRs) and sterol response element binding protein (SREBP), energy sensing mechanisms such as AMP-activated kinase and target of rapamycin (TOR) kinase, as well as endocrine regulators such as insulin (Ashrafi, 2007). The genetic tractability of *C. elegans* allows for the study of metabolic regulation through use of suppressor and enhancer screens to identify components of complex pathways (Hodgkin, 2005).

Thus far, of the more than twenty acyl-CoA synthases encoded by the *C. elegans* genome, phenotypes have been reported for only a few: loss of function mutations in *acs-20* and *acs-22* cause defective cuticle formation (Kage-Nakadai et al., 2010), while *acs-4* and *acs-5* are required for serotonin induced fat reduction (Srinivasan et al., 2008). In this study, we show that reduction of function of *acs-3*, encoding a long-chain acyl-CoA synthase, causes altered patterns of neutral lipid deposition and increased rates of fatty acid uptake and *de novo* synthesis. We show that *acs-3* function in epidermal seam cells is sufficient for restoration of wild type lipid deposition to *acs-3* mutants. Since seam cells are anatomically distinct from sites of fat uptake, *de novo* synthesis, and storage, our findings suggest that *acs-3* must exert its effects on whole animal fat metabolism through mediators that can act cell non-autonomously. To uncover these mechanisms, we conducted suppressor screens and found that the *acs-3* mutant phenotypes could be reverted to wild type by additional mutations in metabolic enzymes that consume acyl-

CoAs and by *nhr-25*, the only *C. elegans* member of the NR5A family of nuclear hormone receptors that includes mammalian steroidogenic factor-1 (SF-1) and liver receptor homolog-1 (LRH-1). Our genetic and biochemical analyses suggest a model whereby ACS-3 generated products ultimately modulate the function of NHR-25, which in turn could regulate an endocrine program of fat uptake and synthesis.

Results

***ft5* mutants exhibit altered fat storage**

To identify genes important in the regulation of fat storage in *C. elegans*, we performed an unbiased mutagenesis screen, and isolated mutants with elevated staining of Nile Red, a solvatochromatic vital dye that has been used in mammalian, *Drosophila*, *C. elegans* and other experimental systems to identify and characterize metabolic pathways involved in fat metabolism (Chen et al., 2009; Flynn et al., 2009; Fowler and Greenspan, 1985; Jones et al., 2008; McKay et al., 2003; Siloto et al., 2009; Suh et al., 2007; Van Gilst et al., 2005). We found a recessive mutant, *ft5*, that exhibited dramatically elevated staining, but developed at nearly the same rate as wild-type animals (Figure 3-1A). To verify that the phenotype was not specific to the Nile Red dye, we grew animals on bacteria containing BODIPY labeled fatty acid, and observed a similar increase in staining intensity (Figure 3-1A). Additionally, *ft5* mutants exhibited large intestinal granules not seen in wild type animals. These enlarged granules were visible with DIC microscopy (Figure 3-1B), stained with BODIPY-labeled fatty acids (Figure 3-1B) and stained in fixed animals with Sudan Black (Figure 3-1F), a diazo dye used for detection of lipids (Greer et al., 2008). They were also encircled by a GFP reporter fused to *C. elegans* ATGL, a lipase that in mammalian and *Drosophila* cells is important for

hydrolysis of triglycerides from lipid droplets (Grönke et al., 2005; Zimmermann et al., 2004) (Figure 3-1C). Similar morphological and ATGL localization phenotypes were recently described in *C. elegans* with increased fat accumulation due to disrupted peroxisomal fat breakdown (Zhang et al., 2010).

Unlike BODIPY-labeled fatty acids or dyes used for detection of lipids in fixed samples, an advantage of Nile Red is that its spectral properties are exquisitely sensitive to the polarity of the environment in which it resides (Fowler and Greenspan, 1985; Greenspan and Fowler, 1985). For instance, in relatively polar phospholipid rich environments, Nile Red's fluorescence emission spectra exhibits a peak intensity at 629 nm while in relatively neutral lipid rich environments such as triglyceride rich fat droplets, it emits yellow-gold fluorescence characterized by an intensity peak at 576 nm (Greenspan and Fowler, 1985). Because of this property, Nile Red has been used in mammalian systems to distinguish among various particles that stain with lipophilic dyes to specifically define neutral lipid rich deposits (Fowler and Greenspan, 1985). Nevertheless, use of Nile Red as a vital dye to interrogate *C. elegans* fat storage has recently been criticized, in part, due to the claim that this dye fails to stain lipid depots in tissues such as the skin-like hypodermis or in developing embryos within the hermaphrodite gonad (Brooks et al., 2009; O'Rourke et al., 2009). We found that by simply increasing the concentration of Nile Red fed to living animals it was possible to visualize yellow fluorescence in the enlarged droplets seen in *ft5* mutants (Figure 3-1D) as well as in hypodermis and developing embryos (data not shown). Moreover, the yellow fluorescent granules emitted with peak intensity between 570-580 nm, indicative of an environment rich in neutral lipids (Figure 3-1E).

The *ft5* mutation maps to a seam-cell expressed acyl-CoA synthase

To identify the causative mutation in *ft5* we performed positional cloning followed by sequence analysis and found a G to A mutation in the T08B1.6 gene. This mutation results in a glycine to glutamic acid substitution at amino acid 118 of the predicted protein (Table 3-1 and 3-S9). T08B1.6 encodes a predicted long-chain fatty acyl-CoA synthase, which we named *acs-3*.

To begin characterization of *acs-3*, we first generated transgenic animals carrying 2.5kb of the *acs-3* upstream regulatory sequence fused to a GFP reporter. GFP expression was observed beginning during embryogenesis and continuing through adulthood. From larval stage L1 to L4, expression appeared restricted to epidermal seam cells, the excretory cell, vulva cells and a subset of unidentified cells in the head and tail (Figure 3-2A, 2B and 3-S1). In addition to prominent seam cell expression, adult animals also exhibited moderate intestinal GFP expression. Tissue expression patterns observed by the promoter-reporter fusion were recapitulated by transgenic animals in which full length *acs-3* cDNA was tagged at the C-terminus with GFP and driven by the same 2.5kb upstream regulatory elements (Figure 3-S1). Nile Red fat staining of these transgenic animals was restored to near wild-type levels indicating that the 2.5kb promoter is sufficient for regulation of lipid storage (Figure 3-2F and 2G).

To determine whether ACS-3 function in a subset of tissues could be sufficient for fat storage regulation, we expressed full length wild-type *acs-3* using a variety of tissue specific promoters in *acs-3(ft5)* mutants. Expression of *acs-3* in seam cells, using the previously characterized *wrt-2* and *grd-10* seam cell specific promoters (Aspöck et al., 1999), was sufficient to almost fully rescue the fat storage phenotype of *acs-3(ft5)*

animals (Figure 3-2F and 2G). We attempted numerous injections of an intestinally driven *acs-3* construct but they caused lethality in nearly all instances. In one case, a single line carrying intestinally expressed *acs-3* was generated, however, the transgenic animals exhibited numerous phenotypes associated with sickness such as slow growth or growth arrest, small body size and clear intestine (data not shown). Expression of *acs-3* specifically in body-wall muscle, a tissue in which we never observed expression with the *acs-3* promoter, failed to alter excess Nile Red staining of *acs-3(ft5)* animals (Figure 3-2F and 2G). Taken together, these data indicated that reconstitution of *acs-3* in seam cells was sufficient to confer wild-type fat staining to *acs-3(ft5)* mutants but expression at similar or higher level in another tissue did not rescue the altered lipid storage of these mutants.

Within the seam cells, expression of full length ACS-3 fused to GFP at the C-terminus driven by the rescuing 2.5kb promoter resulted in localization of the fusion protein throughout the cytoplasm. Expression of the same reporter fusion driven by the seam cell specific promoters *wrt-2*, or *grd-10*, which allowed for nearly full rescue of *acs-3(ft5)* fat storage phenotype, resulted in clear localization of the reporter protein to the plasma membrane of seam cells (Figure 3-2C and 2D). Based on its amino acid sequence, the ACS-3 protein is not predicted to have any transmembrane domains. Thus, ACS-3 is likely a cytosolic enzyme that is localized to the inner leaflet of the plasma membrane of seam cells and the difference in subcellular localization using the indicated promoters is likely a consequence of differences in the extent of promoter activity. Consistent with this, there was no obvious overlap between reporter fusions to ACS-3 and CLK-1, a *C. elegans* mitochondrial protein (Felkai et al., 1999) (Figure 3-S1).

Localization of ACS-3 to seam cells was unexpected, as these cells have not been previously implicated in fat storage or regulation. Seam cells are relatively poorly characterized cells that are part of the skin-like epidermis of *C. elegans*. During development, seam cells exhibit stem cell characteristics as they go through multiple rounds of division whereby a subset of daughter cells regenerates the seam cell population after each division, while the remainder differentiate into epidermal, glial or neuronal cells (Sulston and Horvitz, 1977).

Recombinant ACS-3 exhibits acyl-CoA synthetase activity *in vitro*, and prefers long chain fatty acid substrates

To confirm the predicted function of ACS-3, we generated and purified recombinant protein. Using an *in vitro* colorimetric assay, we tested the activity of this recombinant ACS-3 protein, along with a commercially available *Pseudomonas* ACS as a positive control. *Pseudomonas* ACS exhibited strong ACS activity, and could use a broad range of fatty acid chain lengths as substrate, in accordance with previous findings (Hosaka et al., 1981; Knoll et al., 1994) (Figure 3-S2). Recombinant *C. elegans* ACS-3 also exhibited ACS activity, but was capable of activating a narrower range of fatty acid chain lengths. 18-carbon fatty acid was the preferred substrate chain length, but the enzyme exhibited significant activity on 20- and 22-carbon fatty acids as well (Figure 3-S2). These findings confirm the predicted long-chain fatty acyl-CoA (LCFA-CoA) synthase function of ACS-3.

We next tested the activity of recombinant mutant ACS-3 protein in which we introduced the Glu 118 to Gly mutation identified in *acs-3(ft5)* animals. Surprisingly, this protein exhibited activity equivalent to wild-type protein (Figure 3-S2). Thus, we asked

whether the Glu118 to Gly mutation disrupts other aspects of ACS-3 function such as its localization or expression pattern. As assessed by the full-length GFP reporter, the mutant ACS-3 protein mislocalized to the nucleus (Figure 3-2E). The incorrect trafficking of the mutant protein raised the possibility that the mutant phenotype may arise from inappropriate ACS activity in the seam cell nucleus. However, the recessive nature of the *ft5* mutation, coupled with the fact that *acs-3(ft5)* mutants can be rescued by expression of the wild-type cDNA, indicated that the mutant phenotype corresponded to *acs-3* loss of function rather than neomorphic activity. Thus, the Glu118 to Gly mutation likely disrupts ACS-3 function by mislocalizing this enzyme to the nucleus.

Measures of metabolic parameters demonstrate that *acs-3(ft5)* animals exhibit elevated rates of fatty acid uptake and increased rates of *de novo* fatty acid synthesis

To better understand the altered fat storage phenotype of *acs-3(ft5)* animals, we assayed various behavioral and metabolic parameters of these mutants. *C. elegans* intake nutrients through pharyngeal pumping, the rate of which is subject to modulation by food availability and food quality, as well as experience of starvation prior to food exposure (Avery and Horvitz, 1990; You et al., 2008). We found that pumping rates in *acs-3(ft5)* and wild-type animals were equivalent. Similarly, brood size and rates of movement of *acs-3(ft5)* were indistinguishable from those of wild-type animals (Figure 3-3B, 3C and data not shown). Finally, we found that under conditions of full starvation *acs-3* mutants tended to survive slightly longer than wild type animals, although the differences in overall survival rate were not statistically significant (Figure 3-S1).

The requirement of ACS enzymes for consumption of fatty acids suggested defective β -oxidation as a likely explanation for the observed fat storage phenotype. To

directly test this hypothesis, we fed animals radiolabeled oleic acid and measured rates of production of labeled water, a byproduct of fat oxidation. Surprisingly, *acs-3(ft5)* animals showed an increase in β -oxidation rate (Figure 3-3A). Since this assay is dependent on uptake of radiolabeled oleic acid by whole animals, it raised the possibility that the observed enhanced rate of labeled water production could reflect increased rate of fatty acid absorption rather than increased fat oxidation. To evaluate this possibility, we adapted an assay for fatty acid uptake that has been previously utilized in yeast and mammalian cell culture based experiments (Jia et al., 2007; Li et al., 2005a; Schaffer and Lodish, 1994) as well as recently in *C. elegans* (Spanier et al., 2009).. We empirically determined conditions whereby feeding animals a short pulse of the fluorescently labeled fatty acid C1-BODIPY 500/512 C12 allowed for comparison of rate of fatty acid uptake. Based on this assay, *acs-3(ft5)* animals exhibited a striking increase in uptake rate compared to wild-type animals (Figure 3-3D). After uptake of a pulse of fluorescently-labeled fatty acids, the rate of diminishment of fluorescence was similar in wild-type and mutant animals (data not shown), indicating that the increased accumulation of labeled dietary fatty acids in *acs-3(ft5)* animals reflected increased rate of fat uptake rather than reduced efflux. Assuming that the assay conditions did not saturate the capacity of β -oxidation enzymes, the increased rate of β -oxidation of *acs-3(ft5)* mutants likely reflects increased rate of labeled oleate uptake. Thus, when normalized to the rate of fatty acid uptake, the rate of β -oxidation of *acs-3(ft5)* animals may be similar to that of wild-type animals.

Next, we assessed rates of *de novo* fatty acid synthesis. *acs-3(ft5)* animals exhibited approximately 50% increased rates of synthesis relative to wild-type animals

(Figure 3-3E). To determine whether either the increased rate of uptake or synthesis would alter composition of fats, we examined the fatty acid profile of wild-type and *acs-3(ft5)* mutants. There were no significant differences in fatty acid profiles between these strains (Figure 3-3F).

Together, these data indicated that the altered fat storage of *acs-3(ft5)* animals is not a consequence of altered behaviors or defective fat oxidation, but rather *acs-3(ft5)* mutants exhibit a metabolic shift favoring an increased rate of dietary fatty acid uptake and an elevated rate of *de novo* fat synthesis, processes consistent with the lipid storage phenotype of these animals.

Loss of function of acyl-CoA consuming enzymes suppress the effects of *acs-3(ft5)* mutation

Seam cells are anatomically distant from the intestinal lumen where uptake of dietary fatty acid occurs. Moreover, many genes that encode components of lipid metabolism are expressed in intestinal and epidermal cells, while few have thus far been reported to be expressed in seam cells (Hunt-Newbury et al., 2007; Srinivasan et al., 2008). To elucidate how loss of ACS-3 activity in seam cells elicits the observed whole animal metabolic changes, we sought to identify additional genes required for the observed phenotypes of *acs-3(ft5)* mutants. To do so, we took advantage of the fortuitous observation that treatment of animals with 110 μ M of LY294002, a broad spectrum inhibitor of PI-3 kinases, results in developmental arrest in *acs-3(ft5)* yet causes only minor developmental delay of wild-type animals (Figure 3-4A). LY294002 has been reported to functionally inhibit AGE-1, the sole *C. elegans* class-I PI-3 kinase (Babar et al., 1999). However, we found that growth arrest of drug treated *acs-3* mutants was

unlikely to be only due to inhibition of AGE-1 since *acs-3(ft5); age-1(hx546)* double mutants did not arrest in the absence of drug treatment, nor were they sensitive to treatment with LY294002 (Figure 3-S3). Despite the fact that we did not know the targets through which LY294002 caused growth arrest in *acs-3(ft5)* mutants, the striking arrest phenotype provided a facile screening strategy to search for potential suppressors of *acs-3(ft5)*. We anticipated that such an approach would be more specific than screening for suppression of the *acs-3(ft5)* Nile Red staining phenotype, as inactivation of many genes is known to alter Nile Red staining (Ashrafi et al., 2003).

F2 progeny of mutagenized *acs-3(ft5)* animals were grown on 110 μ M LY294002, and animals that developed to adult stage in 72 hours, a time point at which *acs-3(ft5)* animals are 100% arrested in the L2 or L3 larval stages, were selected (Figure 3-4A). Newly identified *acs-3(ft5)* suppressors were assayed for Nile Red staining independent of LY294002 treatment. In all cases, high Nile Red staining of *acs-3(ft5)* animals was reduced to nearly wild-type levels (Figure 3-4C and 4D) suggesting that the identified mutations indeed suppressed the effects of *acs-3(ft5)*, at least on Nile Red staining, rather than simply preventing LY294002 action.

We identified the molecular lesions in *ft8*, *ft11*, *ft12*, and *ft14* suppressor lines (Table 3-1). In *ft8* and *ft11*, we found missense mutations in the Y45F3A.3 gene, encoding a predicted very long-chain acyl-CoA dehydrogenase, which we named *acdh-11*. In *ft11* we found a missense mutation in the T08G2.3 gene, encoding a predicted medium chain acyl-CoA dehydrogenase, which we named *acdh-10*. Finally, in *ft14*, we identified a nonsense mutation in the F41H10.8 gene, encoding the fatty acid elongase *elo-6* (Table 3-1 and Figures 3-S9 and S10). Identified mutations in *acdh-10*, *acdh-11*,

and *elo-6* suppressed *acs-3(ft5)* in a recessive manner, indicating they were loss of function mutations. The early STOP in *elo-6(ft14)* was consistent with a loss of function, and treatment of *acs-3(ft5)* mutants with two-generation RNAi of either *acdh-10* or *acdh-11* suppressed LY294002-induced growth arrest of these animals in a similar manner to the mutations identified (data not shown).

A number of observations indicated that losses of each *acdh-10*, *acdh-11*, and *elo-6* specifically counteracted the effects *acs-3(ft5)* reduction of function. First, although the LY294002 induced growth arrest, high Nile Red staining, enlarged droplet size, and the high fatty acid uptake rate of *acs-3(ft5)* animals were either partially or fully reverted to wild type levels by each of *elo-6(ft14)*, *acdh-10(ft11)* and *acdh-11(ft8)*, none of these mutations on their own caused a detectable change in these parameters when compared to wild type animals (Figure 3-4A, 4B, 4C, 4D and 3-S4). Second, *acdh-10(ft11)*, *acdh-11(ft8)*, and *elo-6(ft14)* mutants all developed and grew at or near wild-type rates and exhibited normal pharyngeal pumping rates further indicating that these mutations do not correct the altered fat storage of *acs-3(ft5)* mutants merely due to detrimental effects on animal viability or changes in food intake (Figure 3-S5 and data not shown).

To begin elucidating how the newly identified suppressors counter the effects of loss of *acs-3*, we first investigated their expression patterns. In each case, expression of GFP reporter fusion was first detected during embryonic development and persisted through larval and adult stages (Figure 3-S5 and data not shown). As previously reported (Pauli et al., 2006), we found that *elo-6* expression was largely confined to the intestinal cells and undetectable in the seam cells (Figure 3-S5). Similarly, there was no detectable *acdh-11* expression in the seam cells although expression was clearly visible in many

other cell types including the fat storing hypodermal and intestinal cells (Figure 3-S5). Reporter fusion of *acdh-10* exhibited a broad expression in numerous tissues (Figure 3-S5). Thus, reduction of function of ACS-3 can be ameliorated by deficiencies in metabolic enzymes in tissues other than the seam cells.

Since *elo-6* regulates production of mono-methyl branched fatty acids (mmBCFA) (Kniazeva et al., 2004; Seamen et al., 2009), we tested whether *elo-6(ft14)* suppression of *acs-3(ft5)* is due to diminished mmBCFA levels. As expected, *acs-3(ft5); elo-6(ft14)* double mutants had reduced levels of mmBCFA (Figure 3-S6). However, dietary supplementation with these fatty acids failed to reverse the *elo-6(ft14)* suppression of *acs-3(ft5)* (Figure 3-S7), suggesting low mmBCFA levels do not suppress *acs-3(ft5)*. Additionally, there were no obvious changes in mmBCFA caused by losses of *acdh-10* and *acdh-11* (Figure 3-S6).

A unifying explanation for how mutations in each of *acdh-10*, *acdh-11*, and *elo-6* restore wild type growth and lipid metabolic phenotypes to *acs-3(ft5)* mutants is that each of these enzymes modulates fatty acyl-CoA turnover. As an elongase enzyme, ELO-6 acts on fatty acyl-CoAs, effectively depleting the fatty acyl-CoA pool of shorter chain lengths through production of longer chain fatty acyl-CoAs, while ACDH enzymes are well known to function downstream of ACS enzymes in β -oxidation of fatty acyl-CoAs (Eaton et al., 1996). As such, the deficiency in generation of acyl-CoA products of ACS-3 could be countered by simultaneous deficiencies in enzymes that consume fatty acyl-CoAs. Our findings additionally indicate that these enzymes need not function in the very same tissue.

***acs-3* regulation of fat storage and growth is dependant on the nuclear hormone receptor *nhr-25*, a member of the SF-1 and LRH-1 family.**

How might changes in ACS-3 generated acyl-CoA products in the seam cells cause changes in fatty acid uptake and lipid storage phenotypes in intestinal cells? In addressing this question we were guided by the molecular identity of yet another suppressor of *acs-3(ft5)*. In the suppressor line *ft13* we identified a missense mutation in Y46E12BL.4, a previously uncharacterized gene (Table 3-1 and 3-S11). As in the *acdh* and *elo-6* suppressors, the *ft13* mutation suppressed both the LY294002 induced growth arrest and Nile Red staining phenotypes of *acs-3(ft5)* mutants, without causing obvious changes in growth and staining patterns of otherwise wild type animals (Figure 3-S8). We generated RNAi constructs targeting Y46E12BL.4, and found that knockdown of this gene in *acs-3(ft5)* mutants recapitulated the suppression observed in the mutant background, confirming the identity of the suppressor mutation (data not shown).

Y46E12BL.4 encodes an uncharacterized protein predicted to contain a conserved SPRY domain, most closely related to the SPRY domain of *D. melanogaster* GUSTAVUS, an RNA binding protein with roles in early embryonic development, and the mouse Ssb-1, -2, -3 and -4, proteins whose physiological functions remain poorly understood. Each of these homologous proteins contains both a central SPRY domain as well as a C-terminally encoded SOCS box domain. This latter domain was lacking from the wormbase prediction of Y46E12BL.4. We performed 3' RACE to identify the 3' end of the Y46E12BL.4 cDNA and found that this gene does indeed encode a protein with both an SPRY and a C-terminal SOCS box domain. Therefore, we named this gene *spsb-1* (SPry and Socs Box gene).

Although *C. elegans spsb-1* is uncharacterized, and our attempts at obtaining transgenic animals expression GFP fusions to *spsb-1* were unsuccessful, its homology to GUSTAVUS provided a potential clue to mechanisms through which *acs-3* may exert its effects on metabolism. In a yeast two hybrid screen, *Drosophila* GUSTAVUS was found to interact with FTZ-F1 (Giot et al., 2003), an NR5A NHR family member (Asahina et al., 2000). Similar to *acs-3*, *nhr-25*, the sole NR5A family member in *C. elegans*, is expressed in seam cells. Moreover, in response to as-yet-unidentified signals, *nhr-25* regulates molting, an endocrine program that is initiated in the seam cells but exerts effects throughout *C. elegans* tissues (Asahina et al., 2000; Frand et al., 2005; Kostrouchova et al., 1998). The fact that some NHRs are regulated by lipophilic hormones, coupled with the identities of suppressors of *acs-3*, suggested that fatty acyl-CoA product of ACS-3 may directly or indirectly regulate function of NHR-25 to in turn regulate an endocrine program of fat uptake and *de novo* fat synthesis.

At least 27 *C. elegans nhrs*, including *nhr-25*, are reported to be expressed in the seam cells (identified in a search of www.wormbase.org). We tested whether RNAi mediated inactivation of any of these genes could either recapitulate the *acs-3(ft5)* phenotype, or suppress it. Only RNAi knockdown of *nhr-25*, but none of other NHRs tested, suppressed the high Nile Red staining of *acs-3(ft5)* mutants without altering staining in wild-type animals (data not shown). Unambiguous interpretation of this finding was not possible because of pleiotropic phenotypes such as sterility, partially penetrant lethality and reduced body size (Asahina et al., 2000; Chen et al., 2004), associated with *nhr-25* RNAi treatment. Therefore, we generated *acs-3(ft5); nhr-25(ku217)* double mutants. *ku217* is a hypomorphic allele of *nhr-25* that exhibits reduced

DNA binding activity, yet retains enough activity to allow for relatively normal rates and patterns of development up until the adult stage in a majority of animals, at which point all animals exhibit a severe defect in egg-laying (Chen et al., 2004). Experiments with this allele were performed before the onset of the egg-laying deficient phenotype. Nile Red staining, large droplet formation and fatty acid uptake were significantly reduced in *acs-3(ft5); nhr-25(ku217)* double mutants compared to *acs-3(ft5)*, while *nhr-25(ku217)* alone exhibited wild-type fatty acid uptake, Nile Red staining levels and lipid droplet size (Figure 3-5B, 5C, 5D and 3-S4). Moreover, *nhr-25(ku217)* partially suppressed the growth arrest phenotype of LY294002 treated *acs-3(ft5)* animals (Figure 3-5A). Together, these data suggested that phenotypes caused by *acs-3* reduction of function were dependent on *nhr-25*. Consistent with this interpretation, *nhr-25* is expressed in seam cells, the excretory cell and cells of the developing vulva (Asahina et al., 2000; Chen et al., 2004; Gissendanner and Sluder, 2000) a pattern very similar to that of *acs-3* (Figure 3-2A, 2B and 3-S1).

To further investigate the genetic interaction between *acs-3* and *nhr-25*, we examined a number of *nhr-25* dependant phenotype. As previously reported, we found that *nhr-25(ku217)* animals exhibit partially penetrant sterility, while fertile animals that develop to adulthood exhibit a strong egg-laying defective phenotype caused by improper vulval development (Chen et al., 2004). The vulval development defect was partially corrected by the *acs-3(ft5)* mutation as was the sterility associated with loss of *nhr-25* function (Figure 3-5E). *nhr-25(ku217)* animals also exhibit extranumerary seam cells, however this phenotype was neither suppressed nor enhanced in *acs-3(ft5); nhr-25(ku217)* double mutants (data not shown). Together, these findings provide additional

evidence supporting a genetic regulatory relationship between *acs-3* and *nhr-25* consistent with the possibility that loss of *acs-3(ft5)* is associated with enhanced activity of NHR-25.

The *nhr-25* ligand-binding domain accommodates phosphoinositide lipids containing long chain fatty acids

nhr-25 is the single *C. elegans* member of the NR5A family of NHRs. This family includes *D. melanogaster* FTZ-F1, and mammalian LRH-1 and SF-1. One possible mechanism linking *acs-3* and *nhr-25* was suggested by biochemical studies of SF-1 and LRH-1. While the existence and identity of potential ligands of these mammalian receptors have been debated, these receptors accommodate phospholipids containing long chain fatty acids in their ligand-binding pockets (Krylova et al., 2005; Li et al., 2005b). *In vitro* experiments with mammalian SF-1 have shown that phosphoinositides are efficiently exchanged into the ligand-binding domain, raising the possibility that these lipids may act as ligands for these receptors (Krylova et al., 2005; Sablin et al., 2008).

To investigate potential biochemical similarities between mammalian and *C. elegans* NR5A class of receptors, we generated purified recombinant NHR-25 ligand-binding domain protein. Utilizing a previously described assay (Sablin et al., 2008), we tested the ability of this purified protein to incorporate phospholipids *in vitro*. We found that, as in the mammalian NR5A ligand-binding domains (Krylova et al., 2005), NHR-25 ligand-binding domain could indeed accommodate PI(3,4,5)P₃ and PI(4,5)P₂ (Figure 3-6A and 6B).

The ability of purified NHR-25 to bind PI(3,4,5)P₃ and PI(4,5)P₂ along with the observation that *acs-3(ft5)* mutants exhibit a synthetic growth arrested when treated with

the PI-3 kinase inhibitor LY294002, suggest the possibility that phosphoinositides may act as endogenous regulators of *nhr-25*. Since attachment of fatty acids to the inositol sugar is dependent on availability of fatty acyl-CoAs, our findings are consistent with a model whereby ACS-3 exerts control over NHR-25 activity by determining the chain lengths of fatty acids present on these rings.

Discussion

We found that animals deficient in *acs-3*, encoding an acyl-CoA synthase that preferentially produces LCFA-CoA species, exhibited increased fat uptake, increased *de novo* fatty acid synthesis and altered fat storage, including formation of large lipid droplets. Reconstitution of *acs-3* in the seam cells alone was sufficient to correct the fat storage phenotypes associated with loss of *acs-3*. Since seam cells are a cellular component of *C. elegans* epidermis that are not directly involved in dietary fat uptake and *de novo* fat synthesis, our findings indicated that ACS-3 activity modulates organismal fat metabolism through signals that initiate in the seam cells but ultimately act in other tissues. Consistent with this hypothesis, we found that the metabolic consequences of *acs-3* loss require *nhr-25*, a seam cell expressed NHR previously implicated in the regulation of molting, an endocrine process that links nutrient availability and developmental timing. Our genetic suppressor analyses and biochemical characterization of purified NHR-25 are consistent with a model whereby loss of long chain acyl-CoAs generated by ACS-3 results in aberrant function of NHR-25, which in turn, regulates an endocrine signaling cascade that ultimately modulates dietary fat uptake and *de novo* fat synthesis.

The abnormally large lipid droplets in *acs-3(ft5)* intestinal cells, which can be suppressed by mutations in *acdh-10*, *acdh-11*, *elo-6* or *nhr-25*, are reminiscent of those recently described in animals deficient in peroxisomal β -oxidation (Zhang et al., 2010). As has been shown for the peroxisomal mutants, the *acs-3(ft5)* mutant large droplets stain with BODIPY labeled fatty acids and are ringed by ATGL::GFP transgenically expressed in intestinal cells. We found that these large droplets stain with Nile Red with an emission peak intensity of 570-580 nm, indicating that these large droplets are primarily comprised of neutral lipids. These findings underscore the utility of Nile Red staining as an assay to investigate properties of different lipid storage compartments in intact animals.

It was already known that SF-1 and *nhr-25* are both required for gonadal development and fertility, while both *nhr-25* and LRH-1 are necessary for embryonic development (Asahina et al., 2000; Fayard et al., 2004; Hammer and Ingraham, 1999). Our findings indicate that the elevated fatty acid uptake of *acs-3* mutants is *nhr-25* dependent. Similarly, LRH-1 regulates bile acid production, and is required for efficient intestinal uptake of dietary lipids (Mataki et al., 2007). Finally, beyond its well-recognized role in sex determination, mammalian SF-1 functions in energy balance as a key transcriptional regulator of glucocorticoid synthesis in the adrenal glands (Bakke et al., 2001). Thus, in *C. elegans* as in mammals, an NR5A family receptor modulates metabolic related pathways.

One model consistent with our findings is that ACS-3 functions to generate PI(3,4,5)P₃ and PI(4,5)P₂ species that contain long chain fatty acids, which in turn, act as inhibitory ligands for NHR-25. Crystallographic studies with recombinant mammalian

SF-1 and LRH-1 have identified bacterial phospholipids as fortuitous ligands bound within the ligand pocket of these receptors (Krylova et al., 2005; Li et al., 2005b). A number of other phospholipids can also be accommodated by the ligand pocket of these receptors (Krylova et al., 2005; Li et al., 2005b; Sablin et al., 2008). Among these, phosphoinositides PI(3,4,5)P₃ and PI(3,5)P₂ readily bound the ligand-binding domain and mutations interfering with phosphoinositide binding were found to reduce transcriptional activity of SF-1 (Krylova et al., 2005; Sablin et al., 2008). We found that NHR-25 could bind PI(3,4,5)P₃ and PI(4,5)P₂ under the same conditions reported in experiments with mammalian SF-1 and LRH-1 (Sablin et al., 2008). Moreover, sensitivity of *acs-3* mutants to the PI-3kinase inhibitor LY294002 indicated an *in vivo* regulatory relationship between phosphoinositides and *nhr-25*.

Since mammalian NR5A receptors exhibit constitutive activity in many cell types (Fayard et al., 2004), physiological ligands of these receptors may function to inhibit activity or modulate activity specifically toward subsets of transcriptional targets. Given the preference of ACS-3 for long chain fatty acids, it is possible that its reduction of function may preferentially diminish production of PI(3,4,5)P₃ and PI(4,5)P₂ species containing long chain fatty acids. Our genetic data suggest that such species normally act to inhibit activity of NHR-25 toward mechanisms that promote dietary fat uptake and *de novo* fat synthesis. Consistent with the notion that chain lengths of fatty acids present on various lipid species could modulate activity of NR5A class of receptors, there is evidence that phospholipids with medium chain fatty acids enhance *in vitro* binding of purified SF-1 to a short peptide motif derived from the TIF-2 co-activator, while phospholipids containing long chain fatty acids competitively disrupt this binding (Li et

al., 2005b). However, since acyl-CoAs are substrates for generation of a variety of complex lipid species, we cannot rule out the possibility that ACS-3 derived long chain fatty acyl-CoAs could exert their effects on NHR-25 through mechanisms other than phosphoinositides. Similarly, ACS-3 derived fatty acyl-CoAs may exert their effects on NHR-25 through modulation of potential co-activators of NHR-25 or upstream regulatory proteins.

Identification of *in vivo* physiological lipid ligands of NHRs has been a fundamental challenge in understanding these complex transcriptional master-regulators. Such ligands may be present at very low levels, and may only associate with NHRs under specific physiological conditions, making ligand identification very challenging. Our studies point to ACS enzymes as a previously unappreciated *in vivo* mechanism for regulating the function of the NR5A family of NHRs. Though *in vitro* experiments have demonstrated phospholipids can bind and modulate activity of the NR5A family nuclear receptor SF-1, our data represent the first *in vivo* demonstration that genes acting in lipid metabolism function upstream of this family of receptors.

Future Directions

In this study, we have identified a number of genes functioning in concert to regulate lipid physiology. Based on the identities of the genes involved, and the epistatic relationships among the genes, we have proposed what we believe is a likely model for the relationships among these genes. While the data we have generated support this model, additional experiments could be undertaken to test specific aspects of the model, as well as to expand it.

Potentially the most interesting open question; what is the NHR-25 ligand. A number of potential approaches could be employed to attempt to identify this ligand. Perhaps simplest would be to develop a cell culture based assay for NHR-25 activity that could be used to screen small molecules or lipid libraries for compounds that affect NHR-25 transcription. Identification of a small molecule modulator will not definitively determine the identity of *in vitro* ligands, but could provide information as to the characteristics of such ligands. Utilization of a lipid library would be more likely to identify classes of lipids that can act as ligands. If a particular class was identified, more in-depth biochemical analysis of that lipid class could be conducted in *acs-3* mutants on their own, as well as in combination with the *acdh-10*, *acdh-11* and *elo-6* mutations. Presumably these genes alter the concentration or composition of the NHR-25 ligand, or a subset of the ligands.

With a functional cell culture reporter of NHR-25 transcriptional activity, one could also feed these cells with building blocks for various lipid classes, and determine the effect of these nutrients on NHR-25 activity. For example, fatty acids of varying chain length could be supplied in the media to test the possibility that chain length affects ligand activity. *myo*-Inositol could be supplemented in the media to test the effect of phosphoinositides. Serine, or intermediate metabolites in sphingoid base synthesis could also be tested.

A second approach would be to attempt to pull down transgenically expressed, epitope-tagged NHR-25 from *C. elegans* lysates, and to identify the lipid(s) that are pulled down in the process. Similar approaches have been employed with mammalian NR5A family members. Mass-spec analysis could be performed on the protein-lipid

complex, or lipids could be extracted from the pulled down protein, and then analyzed. A number of variations of this approach may also be feasible. *C. elegans* NHR-25 could be expressed in mammalian cell culture, and pulled down from lysates of these cells, assuming that the lipid class(es) that function as ligands in *C. elegans* are present in mammalian cells. Alternatively, large amounts of NHR-25 ligand binding domain produced in bacterial cells could be immobilized on a column, and *C. elegans* lipid extracts could be applied to the column. This could be followed by either release of the protein, hopefully in complex with lipid(s), or by elution of the bound lipids with an organic solvent. Whatever lipid(s) are identified with such an approach could then be examined in *acs-3* as well as suppressor mutants.

Identification of *acs-3* suppressors via the LY294002 synthetic arrest screen was critical to understanding the pathway. Based on the success of this approach, pursuing it further would likely be fruitful. The original screen could be repeated, perhaps larger, and with better selection. When we performed the first screen, selection was somewhat leaky. It appeared that around the edges of the lawn animals were bypassing the arrest. This issue could be eliminated by seeding the plates with bacteria all the way to the edge of the plate, leaving no unseeded surface area. Additionally, a slightly increased concentration of LY294002 may improve selection somewhat. Perhaps 120 μ M would be sufficient, though using higher concentrations may require a different solvent, or perhaps heating the DMSO to increase the stock solution concentration. Concentrations of DMSO over 1% tend to make animals sick. The screen described in this study identified suppressor mutations in four genes, and two alleles were isolated of only one of these genes. It is likely therefore that additional suppressors could be identified.

Screening for suppressors was successful in identifying genes that act in a manner opposite to *acs-3*. However, it could be very useful to identify additional genes that function in a similar manner as *acs-3* within this pathway. Such genes may include those encoding enzymes required to convert fatty acyl-CoAs into the complex lipid(s) that may function as ligands for NHR-25. We have shown that loss of *acs-3* partially suppresses the egg laying phenotype of *nhr-25(ku217)* mutants. The *egl* phenotype of *nhr-25(ku217)* is highly penetrant, especially at higher temperatures. Therefore, a mutagenesis screen for suppressors of *nhr-25(ku217)* *egl* phenotype could identify genes acting in the same manner as *acs-3* to regulate *nhr-25*.

Another aspect of the pathway that remains unclear is the relationship between *spsp-1* and *nhr-25*. Based on the ability of loss of *spsb-1* function to suppress *acs-3* mutant phenotypes, it appears that SPSB-1 may normally function to activate NHR-25 in some way. How this may occur is unclear. Because of the genomic structure of the *spsb-1* gene, we were unable to determine the expression pattern of this gene. We generated constructs containing a few Kb of upstream regulatory sequence fused to GFP, but these yielded no apparent expression. The gDNA contains a very large first intron, greater than 10 Kb, and we suspect that this region may contain additional regulatory sequence. Cloning of the entire gDNA, including a few Kb of upstream regulatory sequence may be necessary to determine the *spsb-1* expression pattern. We would suspect that the gene is likely expressed in seam cells, which would be required for a direct interaction between SPSB-1 and NHR-25. Beyond determining the expression pattern of this gene, it would be useful to perform some epistasis experiments to clarify the relationship between *spsb-1* and *nhr-25*. For example; does loss of *spsb-1* suppress *nhr-25(ku217)* mutant

phenotypes? RNAi of *spsb-1* using constructs we generated is effective, and could simplify such experiments.

SPSB-1 need not directly interact with NHR-25. This protein could be involved in regulation of NHR-25 ligands, or might even regulate ACS-3. Once the expression pattern of this gene is determined, expression of an epitope tagged version of the protein, followed by pull-down may allow for the identification of SPSB-1 interaction partners. The identification of these proteins would likely help elucidate the role for SPSB-1 in this pathway.

Finally, identification of the downstream pathways regulated by *nhr-25* is important to fully understand the pathway. Our microarray analysis of transcriptional changes caused by RNAi mediated knockdown of *nhr-25* revealed that this protein regulates a very large set of genes, either directly or indirectly (see appendix to chapter 3). Many of these changes may be a response to some sort of cuticular defect. Definitive identification of direct NHR-25 transcriptional targets may provide clues to the mechanism by which *acs-3* and *nhr-25* signaling in the seam cells ultimately regulates intestinal biology. Is this accomplished through production and release of a steroid hormone from seam cells? Are hedgehog-like peptides, many of which are expressed within the seam cells, modulated by *acs-3* and *nhr-25*?

NHR-25 has also been found to interact with components of the β -catenin pathway. It remains possible that the mechanism by which *nhr-25* is regulating intestinal cells do not involve transcriptional changes. Identification of additional *acs-3* pathway components through mutagenesis screens could shed light on such a pathway, if it exists.

It is quite apparent therefore, that a great deal remains unknown regarding this pathway. Biology certainly works in mysterious ways! The many tools available to a *C. elegans* geneticist should be equal to the task of un-unknowning these unknowns.

Methods

Vital dye lipid staining and quantification

Synchronized animals were plated on NGM plates containing OP50 and 50nM Nile Red dye. All lipid staining experiments were conducted at 25°C, as *acs-3(ft5)* animals exhibited the strongest phenotype at this temperature. Animals were grown for 40 hours to the young adult stage. Images were acquired on a Zeiss Axioplan II microscope outfitted with a digital CCD camera. Images shown were obtained using a 16x objective. For quantification, images were collected using a 5x objective. The same circular region of interest (ROI) that encompasses one entire animal was applied to each of these images. The minimum fluorescence value was subtracted from the mean of the ROI to correct for background signal. For each experiment, 8-12 animals of each strain were imaged and analyzed.

For BODIPY staining, a 1mg/ml stock of C1-BODIPY 500/512 C12 (Invitrogen) was added to NGM plates seeded with OP50 at a final dilution of 1:50,000. Images were acquired with a 16x objective as described for Nile Red.

For high Nile Red staining, synchronized L4 animals were plated on seeded NGM plates containing 5uM Nile Red (100x) and allowed to stain for 2-4 hours. Spectral data were collected on a Nikon C1si spectral confocal scope utilizing a 488nm laser for excitation and collecting emitted light at 5nm intervals from 500nm to 660nm.

In vitro assay of ACS function

The *acs-3* cDNA was cloned into the bacterial expression vector pBAD-DEST49 (Invitrogen) allowing for L-arabinose inducible expression of the ACS-3 protein with an N-terminal thioredoxin tag to improve solubility, and a C-terminal 6x His tag. This construct was expressed from *E. coli*, and purified with nickel resin. ACS activity was assayed using an enzyme coupled colorimetric assay, modified for use in 96-well format (Knoll et al., 1994). ACS activity generates acyl-CoA species from free fatty acid and CoA. Bacterial acyl-CoA oxidase in the reaction mix acts on acyl-CoA, generating H₂O₂. Catalase in the assay solution produces formaldehyde from H₂O₂ and methanol. Formaldehyde reacts with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole and IO₄⁻ to generate a purple dye that can be detected at 550 nm. We empirically determined the absorption coefficient of the resulting dye to be $3 \times 10^7 \text{ M}^{-1} \text{ 0.5cm}^{-1}$ and used this to calculate the amount of fatty acyl-CoA produced in each experiment. We generated a standard curve and verified that absorption at 550 nm is linear with acyl-CoA concentration within the range tested. Commercially available *Pseudomonas* ACS protein (Sigma) was used as a positive control. All control assays were performed three times, while experimental assays using ACS-3 protein were performed five times.

Generation of transgenic animals

To assess the expression pattern of *acs-3*, 2.5kb of the upstream regulatory sequence of T08B1.6 amplified by PCR and fused to GFP *unc-54* 3' UTR. This product was injected into N2 adults at 5 ng/μl with a *rol-6* coinjection marker to generate strain KQ341. To examine localization of the ATGL protein, we cloned the C05D11.7a cDNA into a Gateway donor vector (Invitrogen). This was recombined with the *vha-6* promoter

into a Gateway destination vector containing GFP and the *unc-54* 3' UTR for intestinal expression. This construct was injected into *acs-3(ft5)* mutant animals at 5ng/ul with *Ptph-1::GFP* as a coinjection marker to generate strain KQ1982. To rescue *acs-3(ft5)*, and determine subcellular localization of the ACS-3 protein, 2.5kb of the upstream regulatory sequence was cloned into a Gateway Donor vector, recombined with the T08B1.6 cDNA (Open Biosystems) into a Gateway destination vector containing GFP and the *unc-54* 3' UTR, and injected into *acs-3(ft5)* adults at a concentration of 3 ng/μl with *Podr-1::RFP* (gift of C. Bargmann) as a coinjection marker to generate strain KQ519. To determine subcellular localization of the mutant ACS-3 protein, the *ft5* mutation was introduced by site directed mutagenesis into the T08B1.6 cDNA Gateway donor vector, which was recombined with the donor vector containing 2.5kb of T08B1.6 upstream regulatory sequence into a GFP destination and injected into N2 animals at a concentration of 2 ng/μl with a *Pmyo-2::mCherry* coinjection marker to generate strain KQ1332. For seam cell rescue, 1.4kb of upstream regulatory sequence from *wrt-2* (Open Biosystems), or 0.9kb upstream regulatory sequence of *grd-10*, was recombined with the T08B1.6 cDNA into a GFP destination vector, and injected at 5 ng/μl with *Podr-1::RFP* coinjection marker into *acs-3(ft5)* generate line KQ579 (*wrt-2*) and KQ577 (*grd-10*). For body-wall specific expression, 2kb of *myo-3* upstream regulatory sequence (Open Biosystems) was recombined with T08B1.6 cDNA into a GFP destination vector and injected at 5 ng/μl with a *Pmyo-2::mCherry* coinjection marker into *acs-3(ft5)* animals to generate line KQ1260. For intestine specific expression, 2kb of *elt-2* upstream regulatory sequence (Open Biosystems) was recombined with the T08B1.6 cDNA into a GFP destination vector and injected at 5 ng/μl with a *Pmyo-2::mCherry* coinjection marker

into *acs-3(ft5)* animals to generate line KQ1335. To examine potential mitochondrial localization of ACS-3, we generated a construct driving the mitochondrial protein clk-1 (Open Biosystems) with the *wrt-2* seam cell promoter, and fused to mCherry. This construct was injected into *acs-3(ft5)* mutant animals at 5ng/ul with *Ptph-1::GFP* as a coinjection marker to generate line KQ795. To examine potential colocalization, this line was crossed with line KQ579, carrying *Pwrt-2::acs-3::GFP*. To confirm *acs-3* is expressed in seam cells, we generated a *Pacs-3::mCherry* promoter fusion construct utilizing 2.5kb of *acs-3* 5' regulatory sequence, and injected this into strain JR667, which carries an integrated SCM::GFP marker.

Fatty acid uptake assay

We developed an assay similar to that reported by Spanier *et al*, 2009. Synchronized L1 animals were plated on NGM plates seeded with OP50 and grown at 25°C to the young adult stage. The pellet from a saturated OP50 culture was resuspended in an equal volume of S-Basal medium to generate assay solution. To this solution 0.75 µl of a 1mg/ml stock solution of C1-BODIPY 500/512 C12 in DMSO was added per ml. Animals were washed off the plates and rinsed 2x in S-Basal medium. After the final rinse, animals were allowed to settle to the bottom of a 15ml conical tube, then a 30 µl aliquot was added 0.5 ml of assay solution in a foil wrapped 1.5ml tube. The animals were incubated at room temperature for 20 minutes while rotating. Following incubation, animals were allowed to settle to the bottom of the tube for 1 minute, then pipeted onto a slide containing an agar pad and pre-chilled on a cold aluminum block. For quantification of uptake, images were collected using a 5x objective. The same circular region of interest (ROI) that encompasses one entire animal was applied to each of these images.

The minimum fluorescence value was subtracted from the mean of the ROI to correct for background signal. For each experiment, 8-12 animals of each strain were imaged and analyzed.

Measurement of *de novo* fatty acid synthesis and fatty acid composition

Metabolic labeling was performed essentially as described (Perez and Van Gilst, 2008). Briefly, equal amounts of bacteria grown in either LB (^{12}C media) or isogro (98.5% ^{13}C enriched Sigma) were mixed and plated onto agarose plates. For each sample, 30,000 synchronized L1 animals prepared from bleaching gravid adults were added to these plates and grown for 44-48 at 20 $^{\circ}$ C. Lipids were extracted and fatty acids analyzed as described (Perez and Van Gilst, 2008).

Fatty acid oxidation assay

Synchronized L4 animals were washed off plates with 0.9% NaCl, then rinsed three times in 0.9% NaCl. Following washes, animals were incubated for 20 minutes in 0.9% NaCl to allow animals to empty their intestines and then resuspended in S-basal in a final volume of 540 μl . An aliquot (20 μl) was stored at -80 $^{\circ}$ C for subsequent protein determination. [9,10(n)-3H] Oleic acid (GE Healthcare) was added to worms to a final concentration of 20 μM (specific activity 67-93 Ci/mol), complexed to fatty acid free BSA in a 2:1 molar ratio. Samples were incubated on an orbital shaker for one hour at 20 $^{\circ}$ C. Subsequently, TCA was added to a final concentration of 5% w/v. After mixing, samples were centrifuged (14000xg, 10min) and the supernatant (1ml) was transferred to a new microcentrifuge tubes. Phosphate buffered saline (250 μl) and 5M NaOH (100 μl) were added prior to ion exchange chromatography on Dowex 1x8 (200-400 MESH) ion exchange columns (1ml). Samples were eluted using water (1ml) and the amount of

$^3\text{H}_2\text{O}$ in the eluate was determined by scintillation counting. Samples without animals were included as background controls. Addition of NaN_3 (10mM) to wild-type animals inhibited fatty acid oxidation to approximately 10%. In addition, feeding animals with *fadR* bacteria, a strain incapable of taking up fatty acids, did not affect labeled water production, demonstrating that bacterial fatty acid oxidation does not contribute significantly to measured rates (data not shown).

***acs-3(ft5)* suppressor screen**

Synchronized L4 *acs-3(ft5)* animals were mutagenized with 47mM EMS (ethyl methanesulfonate) essentially as described (Brenner, 1974). Mutagenized animals were rinsed and plated on NGM plates containing OP50. After 24 hours, animals were collected and bleached to obtain F1 embryos. These animals were incubated overnight in S-Basal medium to synchronize the population, split into 10 pools and plated on NGM plates with OP50. 40,000 F1 animals were collected 72 hours later, and their embryos were collected by bleach treatment. F2 animals were synchronized overnight in S-Basal, then plated at a high density on 10cm plates containing 110uM LY294002 and OP50. Plates were screened 72-96 hours later, and animals that had reached the gravid adult stage were picked. These lines were rescreened, yielding seven lines exhibiting moderate to strong suppression.

NHR-25 LBD protein purification and ligand exchange

The 6XHIS-NHR-25 LBD (amino acids 309-541 of the NHR-25a spliceform) was cloned into pBH4 bacterial expression vector and expressed in DE3 pLysS *e. coli* and purified by Ni-Trap affinity purification followed by Q-ion exchange chromatography and size excluded into 20 mM Hepes (8.0) with 1 mM EDTA, essentially as previously described

for NR5A receptors (Krylova et al., 2005; Sablin et al., 2008). Di-palmitoyl (C16) PI(4,5)P2 and PI(3,4,5)P3 (Cayman Chemical Co, Ann Arbor, MI) were solubilized in water, then immediately added from a Hamilton syringe to a solution of purified NHR-25 LBD (10 μ M final) at a five-fold molar excess of PIP (50 μ M final) in borosilicate glass tubes. Incorporation of PIPs into NHR-25 LBD was allowed to reach equilibrium while nutating at room temperature for 16 hrs under nitrogen, essentially as previously described for mSF-1 LBD (Sablin et al., 2008). Following determination of the elution profile of NHR-25 LBD alone, the apo-NHR-25 LBD and NHR-25 LBD:PIP species were then separated by MonoQ ion exchange chromatography. Peaks were integrated by AKTA FPLC Unicorn 5.1 software, and the percentage of PIP incorporation calculated by dividing the integrated PIP-incorporated peak by the total area under all NHR-25 LBD peaks.

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Figure Legends

Table 1. Mutations isolated

Molecular identities of mutant alleles identified in this study. *acs-3(ft5)* was isolated from a screen for animals with altered Nile Red staining, while all other mutants were isolated from an *acs-3(ft5)* suppressor screen by identifying animals that do not arrest on 110uM LY294002. Each allele identified in this screen also suppressed the fat storage phenotype of *acs-3(ft5)*.

Figure 3-1. Mutation of *acs-3*, a long chain acyl-CoA synthase, results in altered fat storage.

From a mutagenesis screen we identified a line, *acs-3(ft5)*, that exhibits elevated staining when fed bacteria labeled both with Nile Red (50nM) and BODIPY-labeled fatty acid (A). Further analysis of *acs-3(ft5)* mutants revealed the presence of large refractile droplets visible using DIC microscopy, which were not seen in wild type animals. These large droplets stain with BODIPY-labeled fatty acid (B). The lipase ATGL, a lipid droplet associated protein, appears as rings associated with the perimeter of these large droplets in *acs-3(ft5)* mutant animals (C). When *acs-3(ft5)* mutant animals are labeled with 5 μ M (100x) Nile Red, these droplets stain yellow-gold, while intestinal cells of wild type animals lack these large droplets (D). To measure emission spectra, we utilized a spectral confocal microscope, exciting with a 488nm laser, and collecting emitted light at 5nm intervals between 500nm and 660nm. The emission peak for an individual droplet is between 470-480nm, indicating that these large droplets contain neutral lipids (E). When

fixed and stained with the lipid dye Sudan Black, these large droplets are clearly labeled (F). In all panels, arrows indicate the position of abnormally large lipid droplets. In (C), the scale bar represents 5 μ M and N denotes the position of an intestinal cell nucleus.

Figure 3-2. *acs-3* has a limited expression pattern, and expression of *acs-3* in seam cells rescues the fat storage phenotype.

To examine the expression pattern of *acs-3*, we fused 2.5kb of upstream regulatory sequence to GFP. This construct yields high expression in a limited set of tissues. In L4 animals, strong expression is evident in seam cells, which are beginning to fuse at this stage, the excretory cell (marked Ex) and vulva (marked Vul) as well as cells in the head and tail (A). In an L2 animal, individual seam cells are clearly visible (B). We also generated constructs driving the *acs-3* cDNA fused to GFP. We drove this construct with the previously characterized seam cell promoters *wrt-2* (C) and *grd-10* (D). With both constructs, ACS-3 is clearly localized to the cell membrane (C-D). When we drove the *acs-3(ft5)* mutant cDNA we observed misslocalization of the protein to seam cell nuclei (E). In panels A-E, arrows mark the position of seam cells. Elevated Nile Red staining of *acs-3(ft5)* animals can be rescued by expression of *acs-3* cDNA with regulatory elements upstream of *acs-3* start site, as well as with the *wrt-2* and *grd-10* seam cell specific promoters (E). Expression in the body-wall muscle, a tissue in which *acs-3* is not normally expressed, driven by the *myo-3* promoter, fails to rescue the high Nile Red staining of *acs-3(ft5)* animals (F). We quantified Nile Red intensity in these lines (G). Data are shown as a percentage of the wild-type average $\pm$ SEM. *** $p < 0.001$ compared to *acs-3(ft5)*.

Figure 3-3. Characterization of metabolic parameters reveals *acs-3(ft5)* animals

have an increased rate of fatty acid uptake and elevated *de novo* fatty acid synthesis

We examined a number of physiological parameters that could impact lipid storage. To assess β -oxidation, we incubated animals in media containing tritiated fatty acids, collected that media and measured amount of tritiated water produced (see methods for more details). We found that *acs-3(ft5)* animals exhibited an increased rate of β -oxidation (A). Measures of pharyngeal pumping rate and progeny production revealed no differences between wild type and *acs-3(ft5)* mutants (B-C). To examine fatty acid uptake, we incubated animals in media containing BODIPY-labeled fatty acids for 20 minutes, then measured fluorescence intensity. We found that *acs-3(ft5)* mutants exhibit more than double the rate of fatty acid uptake observed in wild type animals (D). For panels A-D, data are displayed as a percentage of the wild-type average $\pm$ SEM. *** $p < 0.001$ compared to wild-type. Using ^{13}C labeling, we found that *acs-3(ft5)* mutants exhibit a higher percentage of *de novo* synthesized fatty acids (E). ** $p < 0.01$, *** $p < 0.001$ compared to wild type. GC/MS analysis reveals no substantial changes in fatty acid composition between *acs-3(ft5)* mutants and wild type animals (F). Data are shown as a percentage of total fatty acid $\pm$ SEM.

Figure 3-4. An unbiased mutagenesis screen identifies two acyl-CoA dehydrogenases and a fatty acyl-CoA elongase as suppressors of *acs-3(ft5)* growth and fat storage phenotypes.

acs-3(ft5) animals exhibit a developmental arrest when treated with the PI-3 kinase inhibitor LY294002. We performed an unbiased mutagenesis screen utilizing this phenotype, and identified two alleles of *acdh-11*, one allele of *acdh-10* and one allele of *elo-6* as suppressors of this developmental phenotype (A). These mutations also suppressed the high fatty acid uptake of *acs-3(ft5)* mutants, but do not affect uptake in an otherwise wild type background (B). The elevated Nile Red staining phenotype of *acs-3(ft5)* mutants was also suppressed by these mutations, while each of these suppressors, when outcrossed to an otherwise wild type background exhibited normal Nile Red staining phenotype (C-D). Data are displayed as a percentage of the wild-type average +/- SEM. **p < 0.01, ***p < 0.001 compared to *acs-3(ft5)*.

Figure 3-5. Mutation of *nhr-25* suppresses the high Nile Red staining and fatty acid uptake phenotypes of *acs-3(ft5)* animals

The hypomorphic *nhr-25(ku217)* allele partially suppresses the developmental arrest of *acs-3(ft5)* mutant animals grown on LY294002 (A). Arrow indicates position of an arrested *acs-3(ft5)* animal. *nhr-25(ku217)* also suppresses the high fatty acid uptake phenotype and high Nile Red staining phenotype of *acs-3(ft5)* animals, but *nhr-25(ku217)* in an otherwise wild type background exhibits wild type fatty acid uptake and Nile Red staining (B-D). Data are shown as a percentage of the wild-type average +/- SEM. ***p < 0.001 compared to *acs-3(ft5)*. We also tested the affect of *acs-3(ft5)* on previously described *nhr-25(ku217)* phenotypes. We found that loss of *acs-3* function can partially suppress both the sterility and egg-laying phenotypes of *nhr-25(ku217)* (E-F). Data are shown as a percent of all animals assayed. Because the severity of the egg-laying and

sterility phenotypes in *nhr-25(ku217)* is temperature dependant, we performed these assays at 24 degrees, a temperature at which these phenotypes are strong, but most animals develop to adulthood.

Figure 3-6. NHR-25 ligand-binding domain binds phosphoinositides

Mammalian SF-1, an *nhr-25* homolog, has been shown to bind phosphoinositide species. To test the ability of NHR-25 to bind these lipids, we generated and purified recombinant NHR-25 ligand binding domain. Incubation of this protein with PI(4,5)P and PI(3,4,5)P alters retention of the protein in ion exchange chromatography (A) indicating the protein has bound the lipid. Incubation of each phosphoinositide species with NHR-25 ligand binding domain protein induced ~50% of the protein to bind lipid (B).

Figure 3-S1

To confirm that *acs-3* is expressed in seam cells, we generated transgenic animals expressing *Pacs-3::mCherry* and *SCM::nlsGFP*, a well characterized marker of seam cells that localizes to seam cell nuclei. In these animals, seam cells clearly express both cytoplasmic mCherry and nuclear localized GFP (A). We also tested the possibility that the ACS-3 protein is localized to mitochondria. In animals expressing *Pwrt-2::acs-3::GFP* and *Pwrt-2::clk-1::mCherry* a previously characterized mitochondrial protein, no significant colocalization was apparent (B). Autofluorescence is visible as hazy yellow staining. Expression of *Pacs-3::acs-3::GFP* rescues the high Nile Red staining of *acs-3(ft5)* mutant animals (shown in Figure 3-2). These animals express the fusion protein at a very low level, but expression is clearly evident in seam cells as well as cells in the

head of an L2 animal (C). Altered lipid storage could change the ability of an animal to survive starvation, however we observed no significant difference between *acs-3(ft5)* mutants and wild type animals in a starvation assay (D). Error bars indicate SEM.

Figure 3-S2

Using a colorimetric biochemical assay, we tested the acyl-CoA synthase activity of ACS-3 protein. As a positive control we assayed a commercially available *Pseudomonas* ACS, and found that it could utilize a broad range of substrate chain lengths (A). By contrast, ACS-3 protein could only utilize fatty acids 18 or more carbons in length (B). ACS-3 mutant protein exhibits activity equivalent to wild type protein. As a negative control, column eluent from uninduced bacteria was included. Because only one sample was used for each negative control measurement, no error bars are shown. Error bars indicate SEM.

Figure 3-S3

The PI-3 kinase inhibitor LY294002 induces developmental arrest in *acs-3(ft5)* mutant animals. To test whether this arrest is the result of pharmacological inhibition of the PI-3 kinase AGE-1, we generated *acs-3(ft5); age-1(hx546)* double mutants. These double mutants developed normally, and exhibited no sensitivity to LY294002 treatment. This drug may act through one of the other members of the PI-3 kinase family, or perhaps induces developmental arrest by inhibiting multiple kinases.

Figure 3-S4

Nearly all *acs-3(ft5)* animals exhibit large lipid droplets. Using DIC microscopy, we examined wild type, *acs-3(ft5)*, and *acs-3(ft5)* suppressors *acd-11(ft8)*, *acd-10(ft11)*, *elo-6(ft14)* and *nhr-25(ku217)* in an *acs-3(ft5)* background as well as in an otherwise wild type background. We found that *nhr-25(ku217)* reduces the percentage of *acs-3(ft5)* animals exhibiting large droplets to nearly wild type levels. *elo-6(ft14)* also strongly suppresses this phenotype, while *acd-11(ft8)* and *acd-10(ft11)* only modestly suppress. Animals were grown at 24 degrees and assayed at the L4 stage.

Figure 3-S5

acs-3(ft5) suppressors *acd-11* and *acd-10* are broadly expressed. *acd-11* expression in larval stages is apparent in intestine (Int) and hypodermis (H), though not in seam cells (A). In adult stages, *acd-11* expression is primarily intestinal (A). *acd-10* expression appears to be broad, clearly apparent in tissues including pharyngeal muscle (P), intestine and hypodermis (A). Expression of *elo-6* is restricted to intestine, as has been previously described (A). To further examine these suppressor lines, we assayed progeny production and pharyngeal pumping rate (B-C). While all lines exhibited wild type pumping rates, *acd-10(ft11)* exhibited moderately reduced fecundity, and *elo-6(ft14)* exhibited slightly reduced progeny production.

Figure 3-S6

An analysis of the fatty acid profile of each of the lipid metabolic enzyme mutants reveals few significant changes. No significant differences are apparent between wild type animals and *acs-3(ft5)* mutant animals. The only strains exhibiting significant

changes in fatty acid profile are *elo-6* and *acs-3; elo-6*. Both strains have significantly reduced levels of mono methyl branched chain fatty acids C15iso and C17iso. However, dietary supplementation with mono methyl branched chain fatty acids failed to desuppress *acs-3;elo-6* double mutants (figure 3-S7). Error bars show SEM.

Figure 3-S7

Loss of *elo-6* causes reduced levels of mono-methyl branched chain fatty acids, as expected (see Figure 3-S4). We considered the possibility that reduced levels of these fatty acids could underlie *elo-6(ft14)* suppression of *acs-3(ft5)*. If this were the case, dietary supplementation with mono-methyl branched chain fatty acids could desuppress *acs-3(ft5); elo-6(ft14)*, causing these animals to again arrest when treated with LY294002. However, supplementation with either C17iso (shown here) or C15iso (not shown) fails to rescue the *elo-6(ft14)* suppression of *acs-3(ft5)*. Two generation RNAi of the related elongase *elo-5* causes developmental arrest at the L1 stage. Dietary supplementation with C17iso rescues this developmental arrest, confirming that the fatty acid is taken up in this assay.

Figure 3-S8

From the suppressor mutagenesis screen that yielded enzymatic suppressors of *acs-3(ft5)* we also identified a mutation in *spsb-1*. This mutation suppresses the high Nile Red staining of *acs-3(ft5)*, but in an otherwise wild type background does not affect Nile Red staining (A-B). Data are shown as percent of wild type average $\pm$ SEM. *** $p < 0.001$ compared to *acs-3(ft5)*. In addition, *spsb-1(ft13)* strongly suppresses the LY294002

developmental arrest of *acs-3(ft5)* (C). The predicted protein sequence contained an SPRY domain closely related to the fly gene *gustavus* and the mammalian Ssb genes, each of which has an SPRY domain, as well as a C-terminal SOCS box domain. We performed 3' RACE and discovered that *spsb-1* does in fact contain a C-terminal SOCS box domain. The domain structure of SPSB-1 is shown in (D). The asterisk denotes the position of the *spsb-1(ft13)* mutation.

Figure 3-S9

SNP mapping was used to map *acs-3(ft5)* to between -16.95 and -15.88 on chromosome V. *elo-6(ft14)* was mapped to between 1.59 and 1.67 on chromosome IV. Recombinant genotypes are shown.

Figure 3-S10

SNP mapping was used to map *acdh-11(ft8)* to between -0.26 and -3.8 on chromosome III. *acdh-10(ft11)* was mapped to between 16.71 and 24.49 on chromosome X. Recombinant genotypes are shown.

Figure 3-S11

SNP mapping was used to map *spsb-1(ft13)* to the right side of 24.53 on chromosome II. This region contains only 4 genes.

Table 3-1

Allele	Gene ID	Gene Name	Gene Function	Locus	Mutation
<i>ft5</i>	T08B1.6	<i>acs-3</i>	Acyl-CoA Synthase	V:-16.58	Gly118->Glu
<i>ft8</i>	Y45F3A.3	<i>acdH-11</i>	Acyl-CoA Dehydrogenase	III:3.73	Asp309->Asn
<i>ft12</i>	Y45F3A.3	<i>acdH-11</i>	Acyl-CoA Dehydrogenase	III:3.73	Ala244->Thr
<i>ft11</i>	T08G2.3	<i>acdH-10</i>	Acyl-CoA Dehydrogenase	X:24.09	Gly259->Glu
<i>ft14</i>	F41H10.8	<i>elo-6</i>	Fatty Acid Elongase	IV:1.64	Gln205->STOP
<i>ft13</i>	Y46E12BL.4	<i>spsb-1</i>	Unknown	II:24.58	Gly3->Glu

Figure 3-1

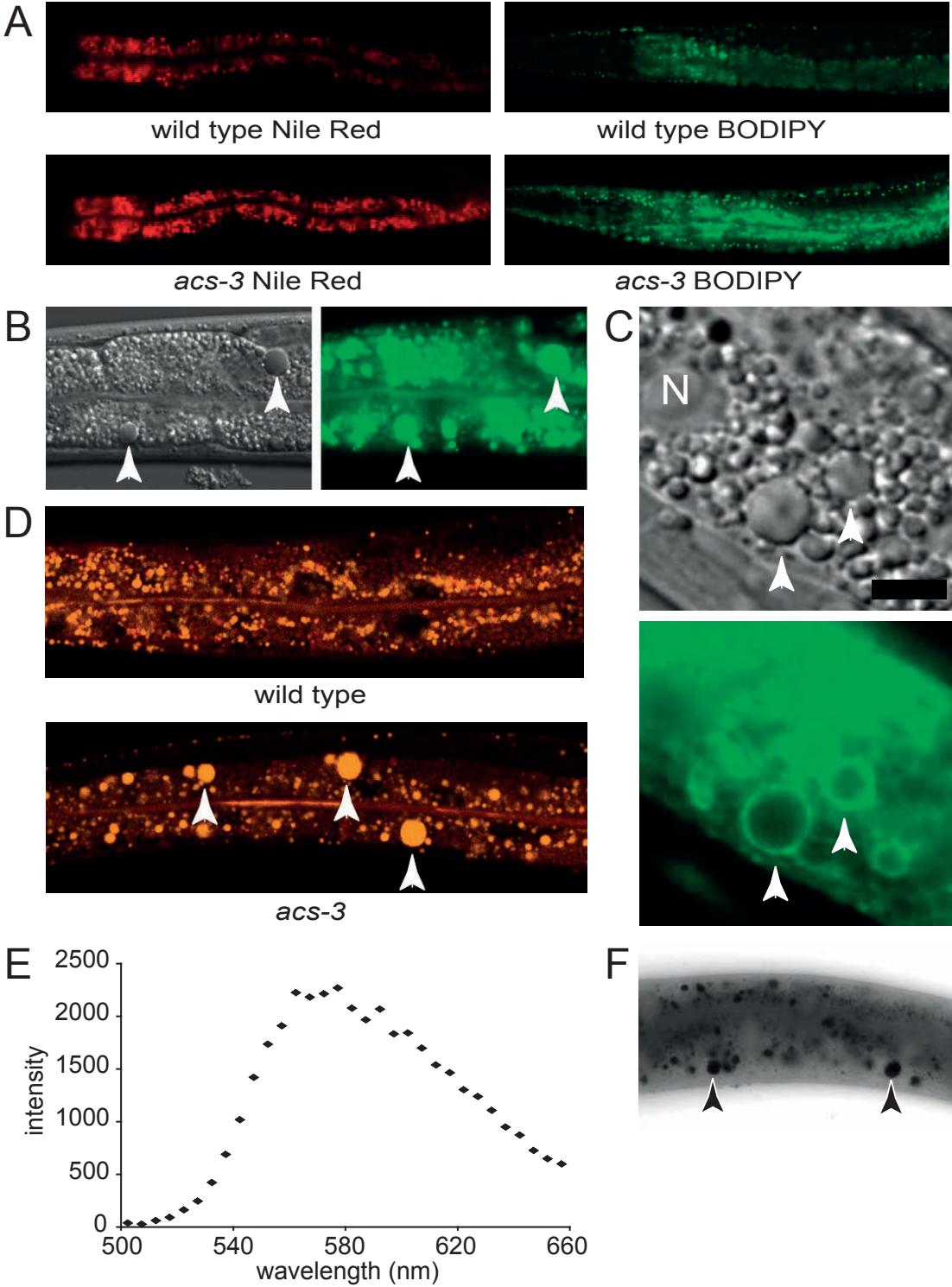


Figure 3-2

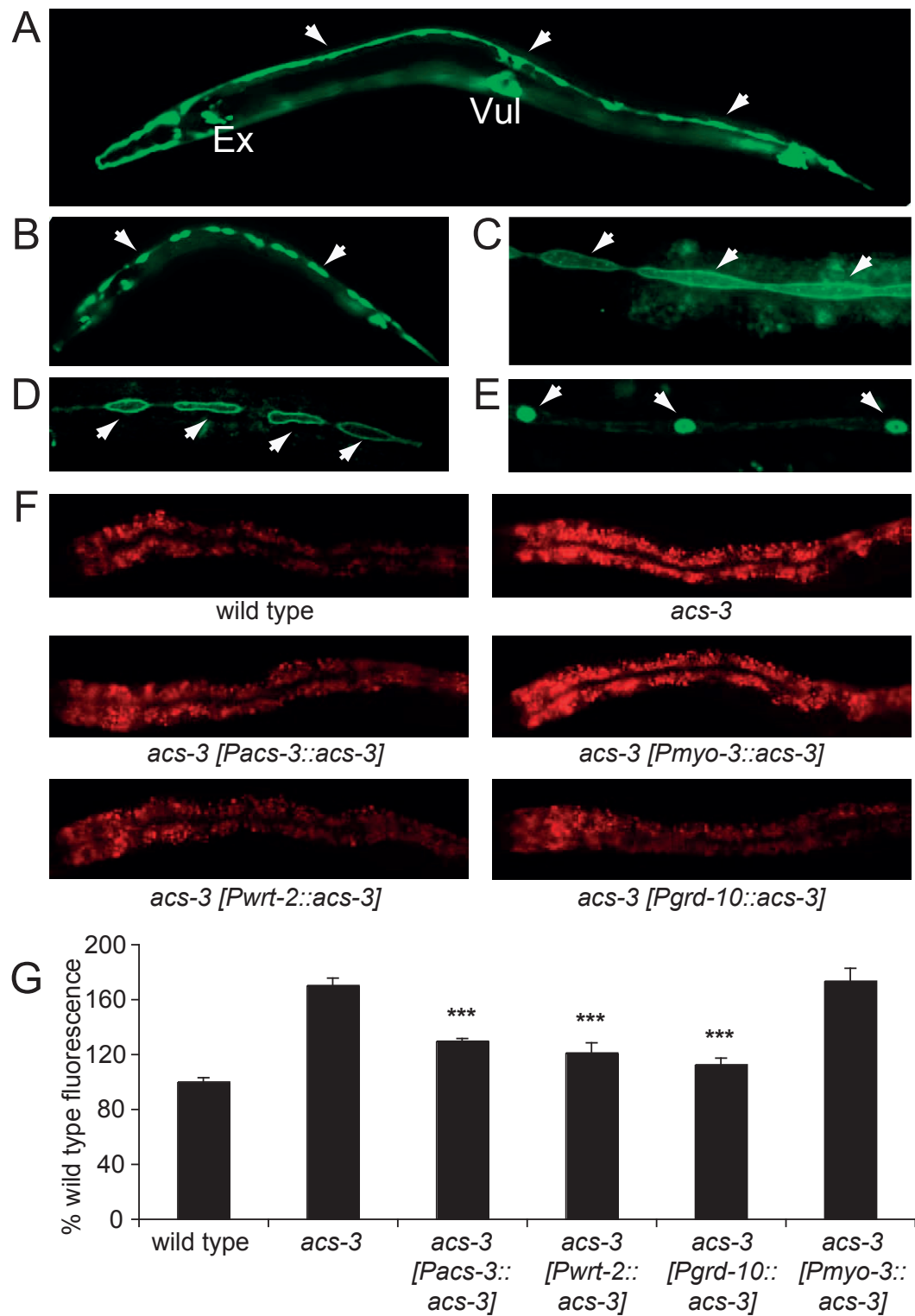


Figure 3-3

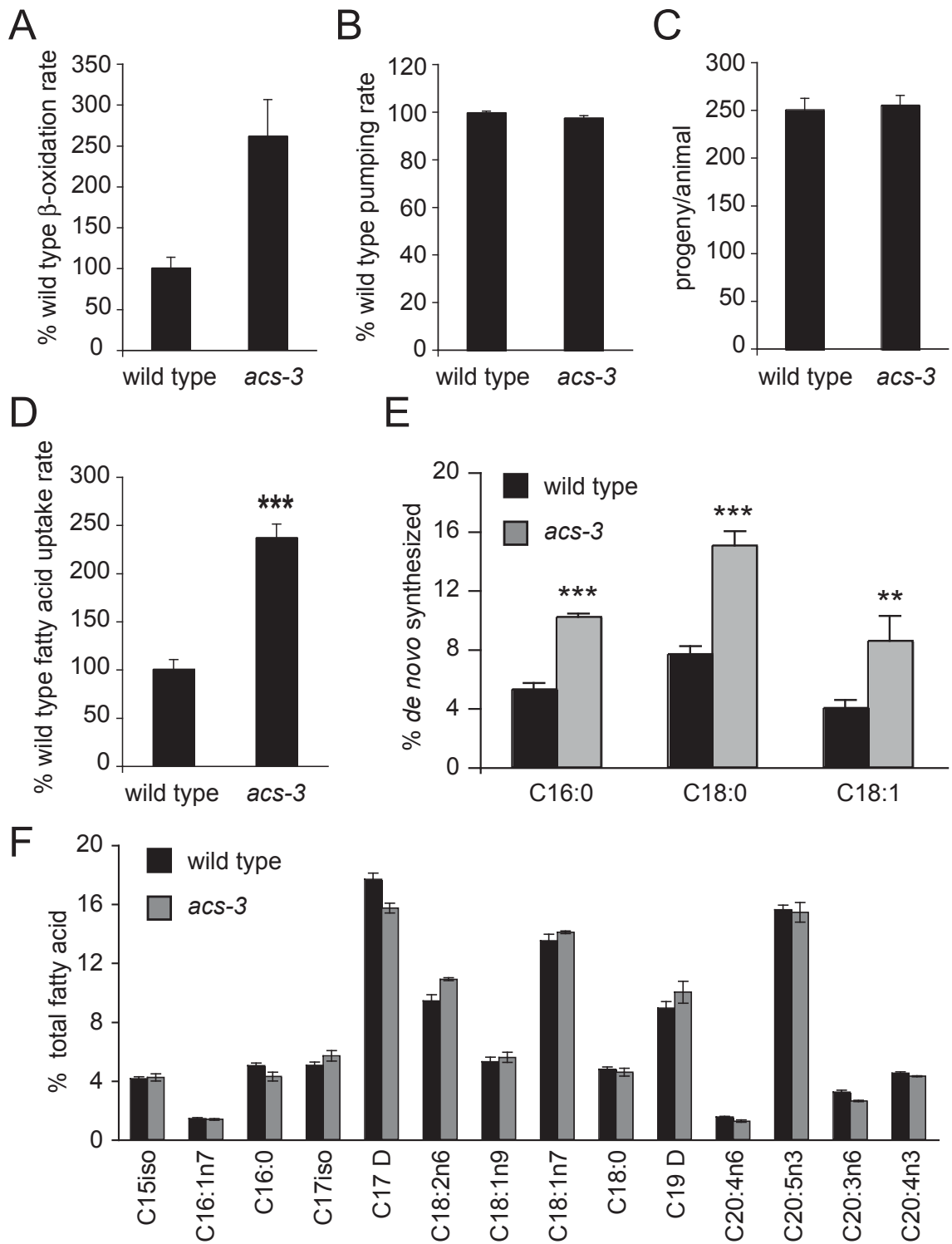


Figure 3-4

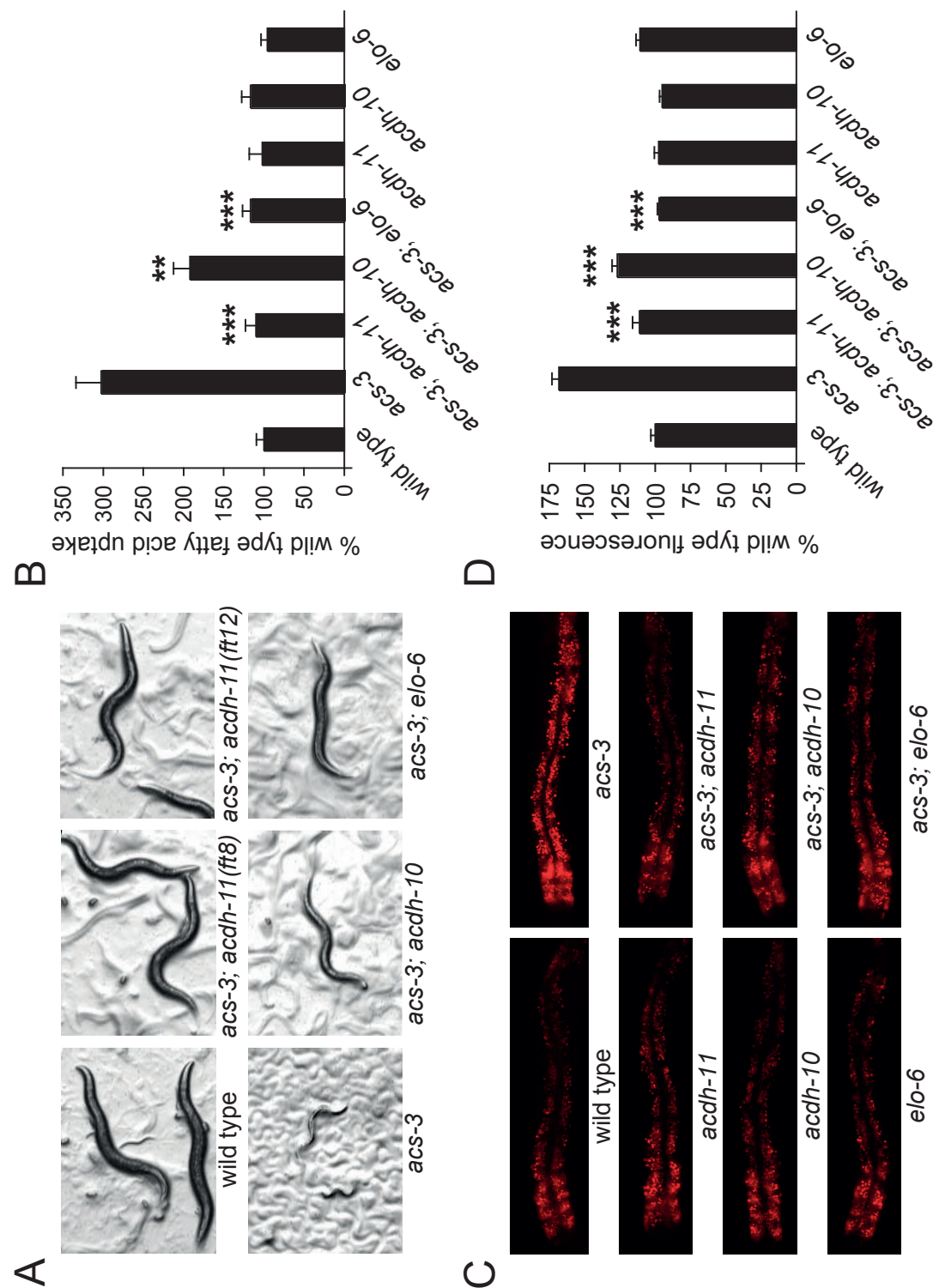


Figure 3-5

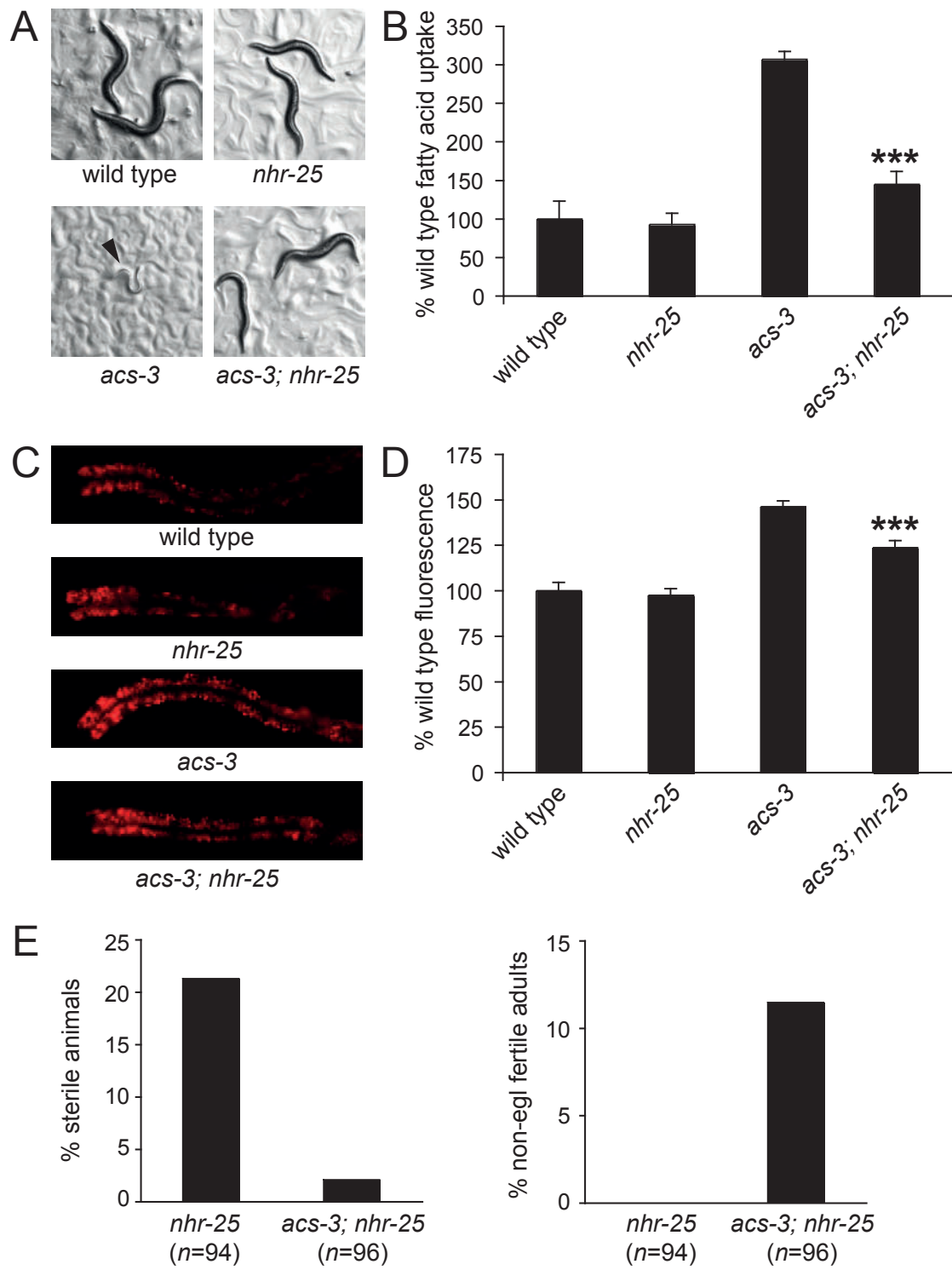


Figure 3-6

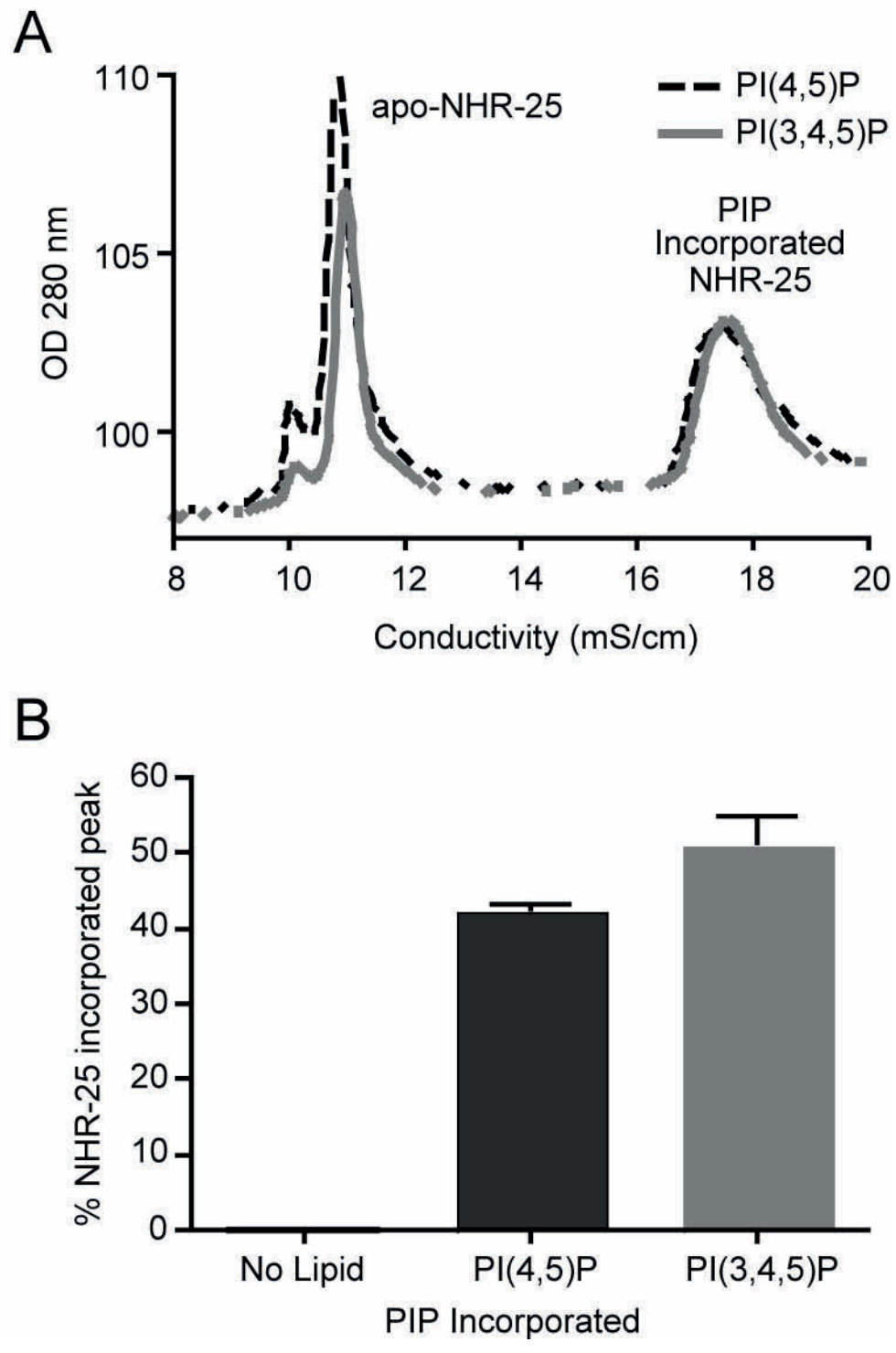


Figure 3-S1

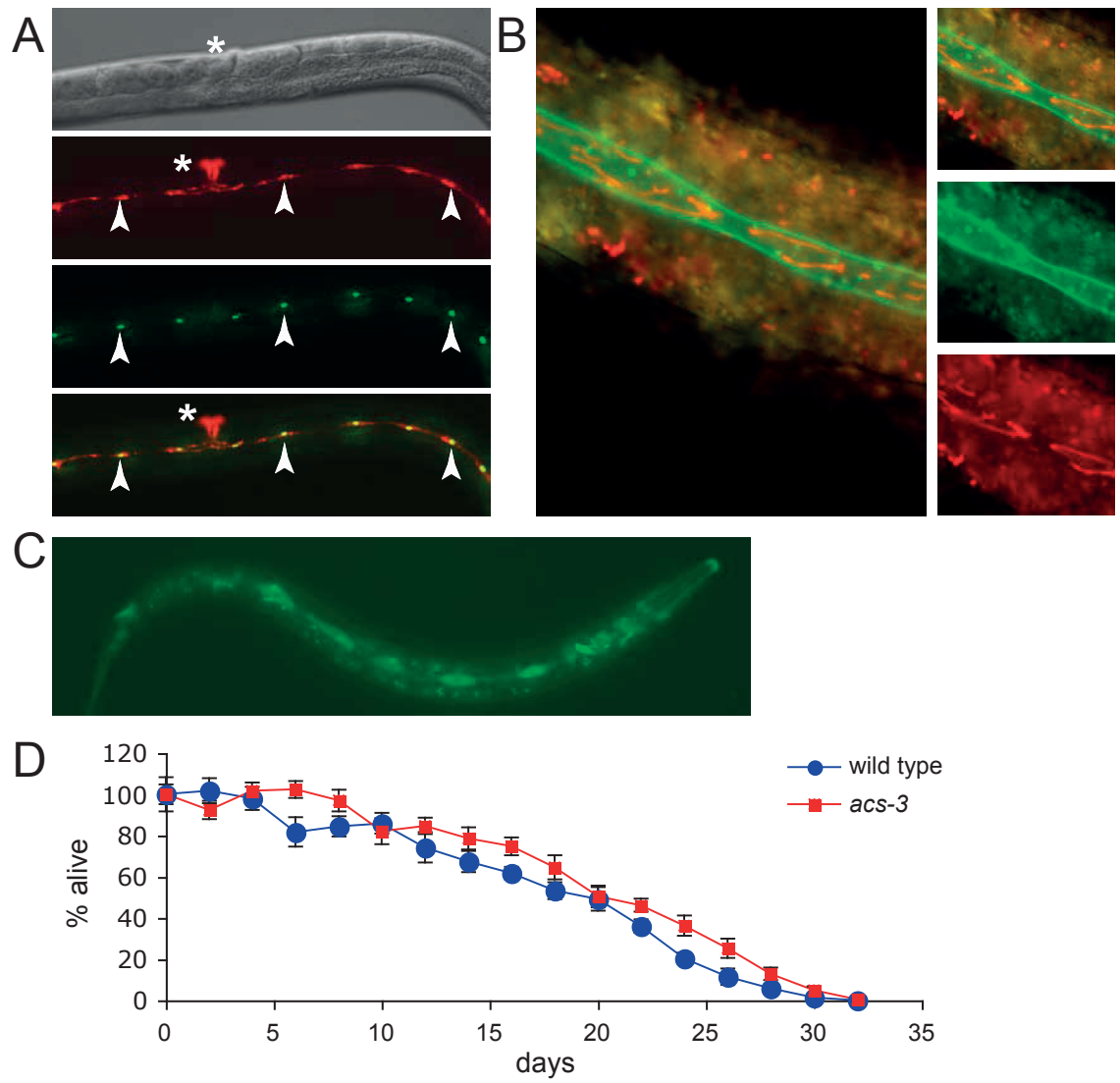


Figure 3-S2

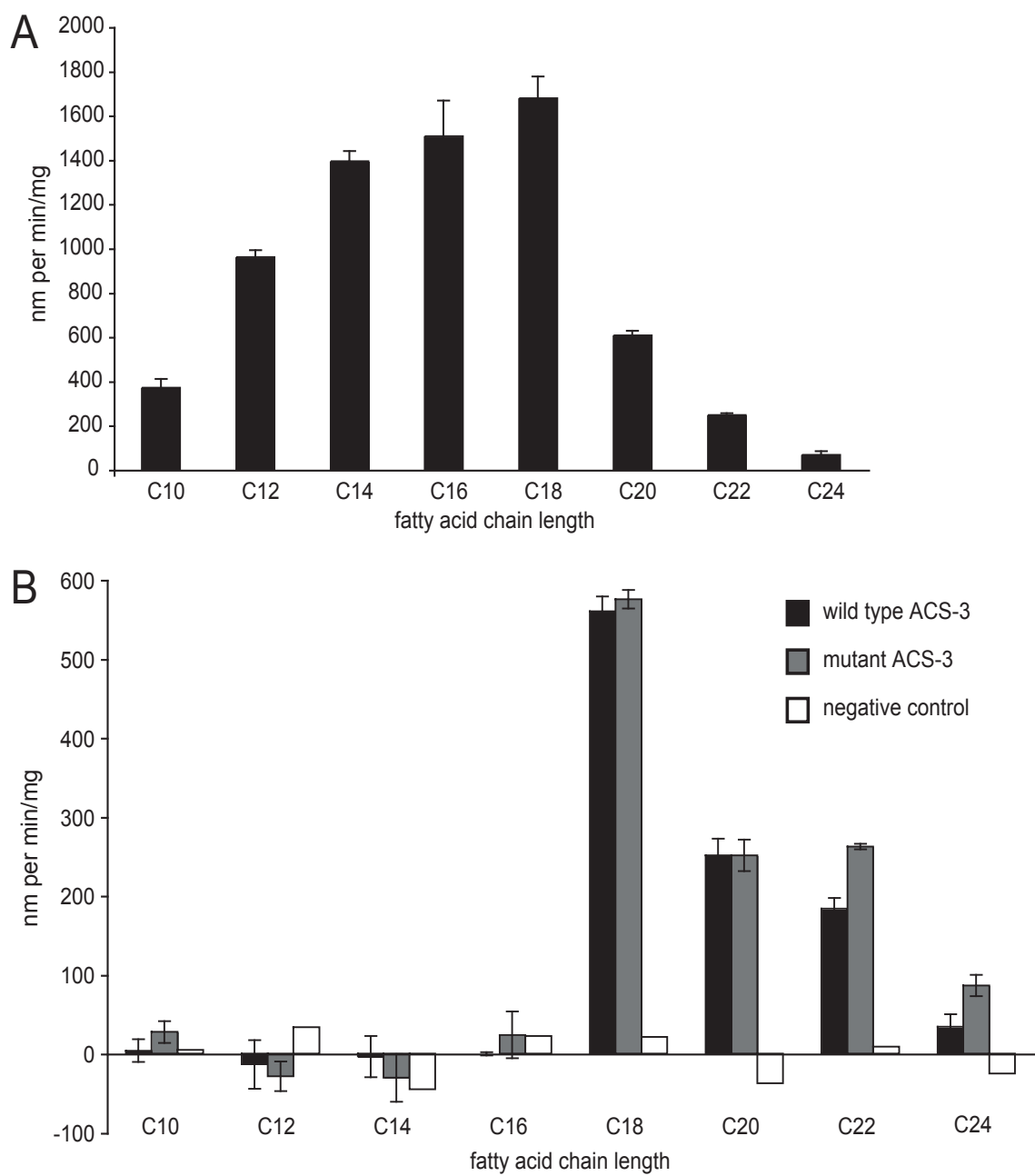


Figure 3-S3

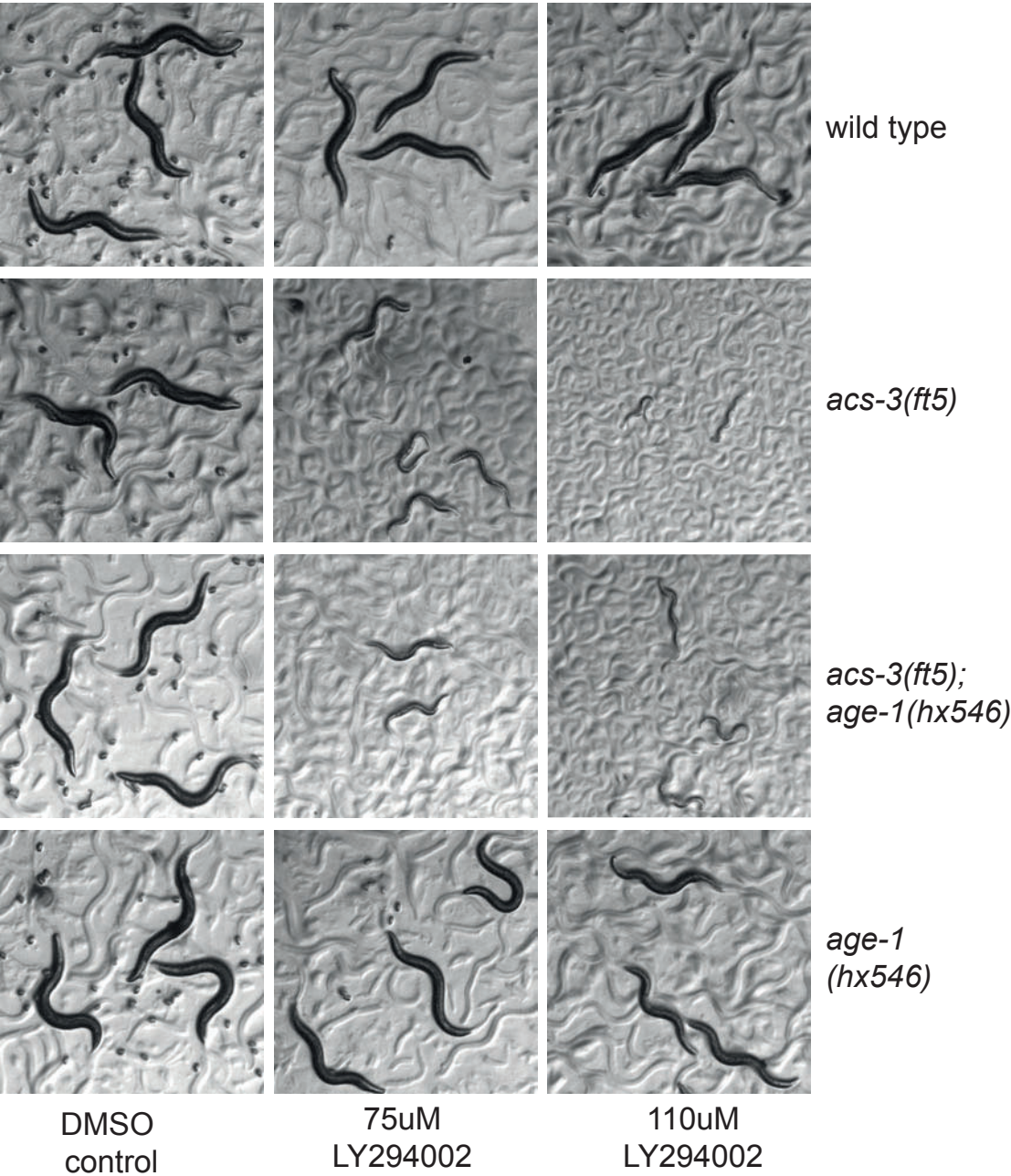


Figure 3-S4

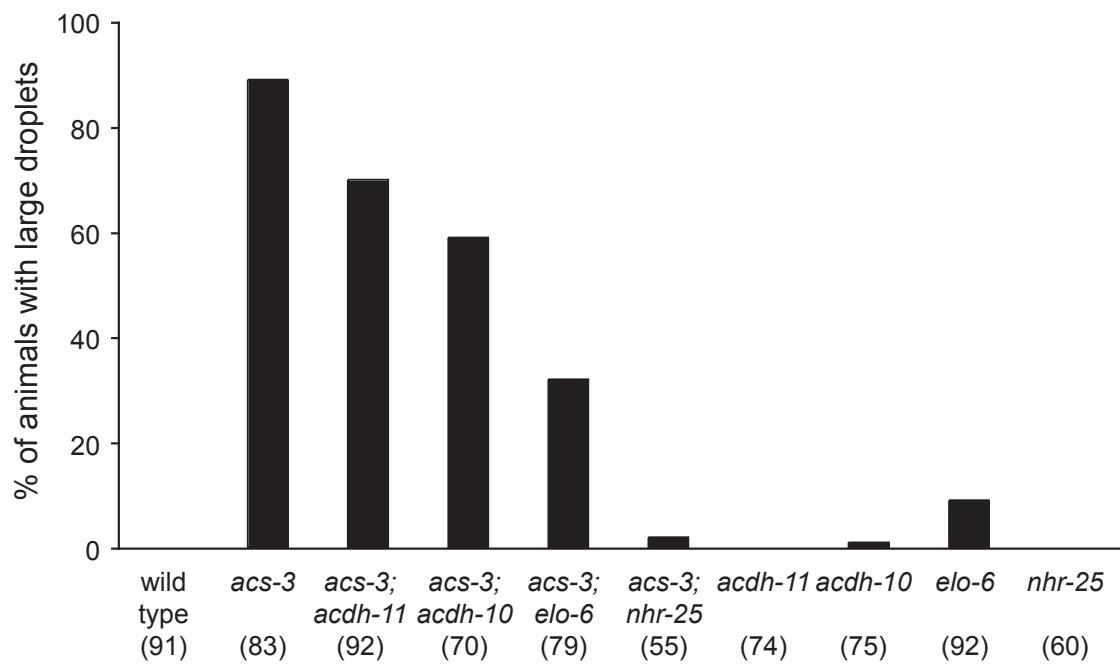


Figure 3-S5

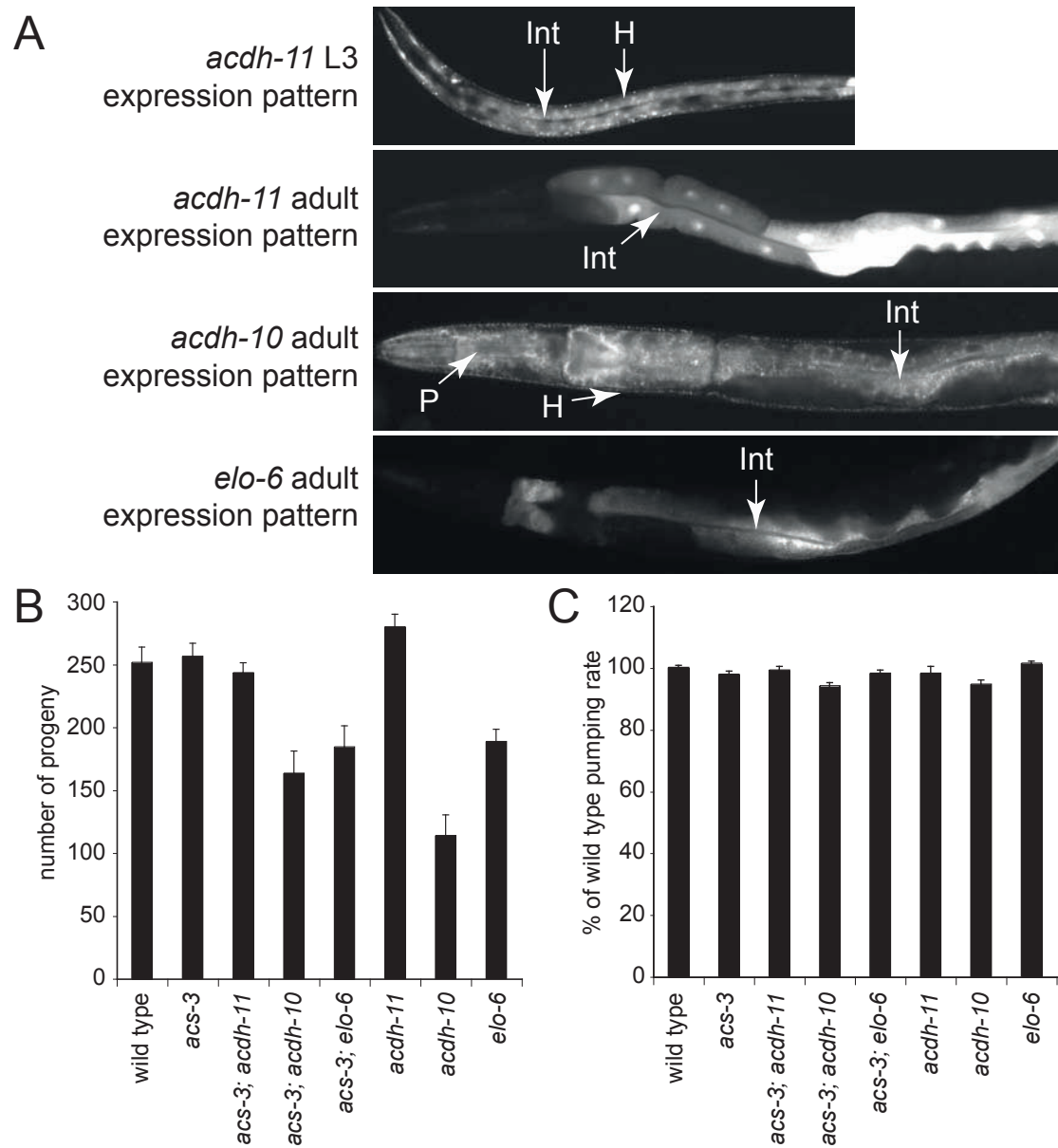


Figure 3-S6

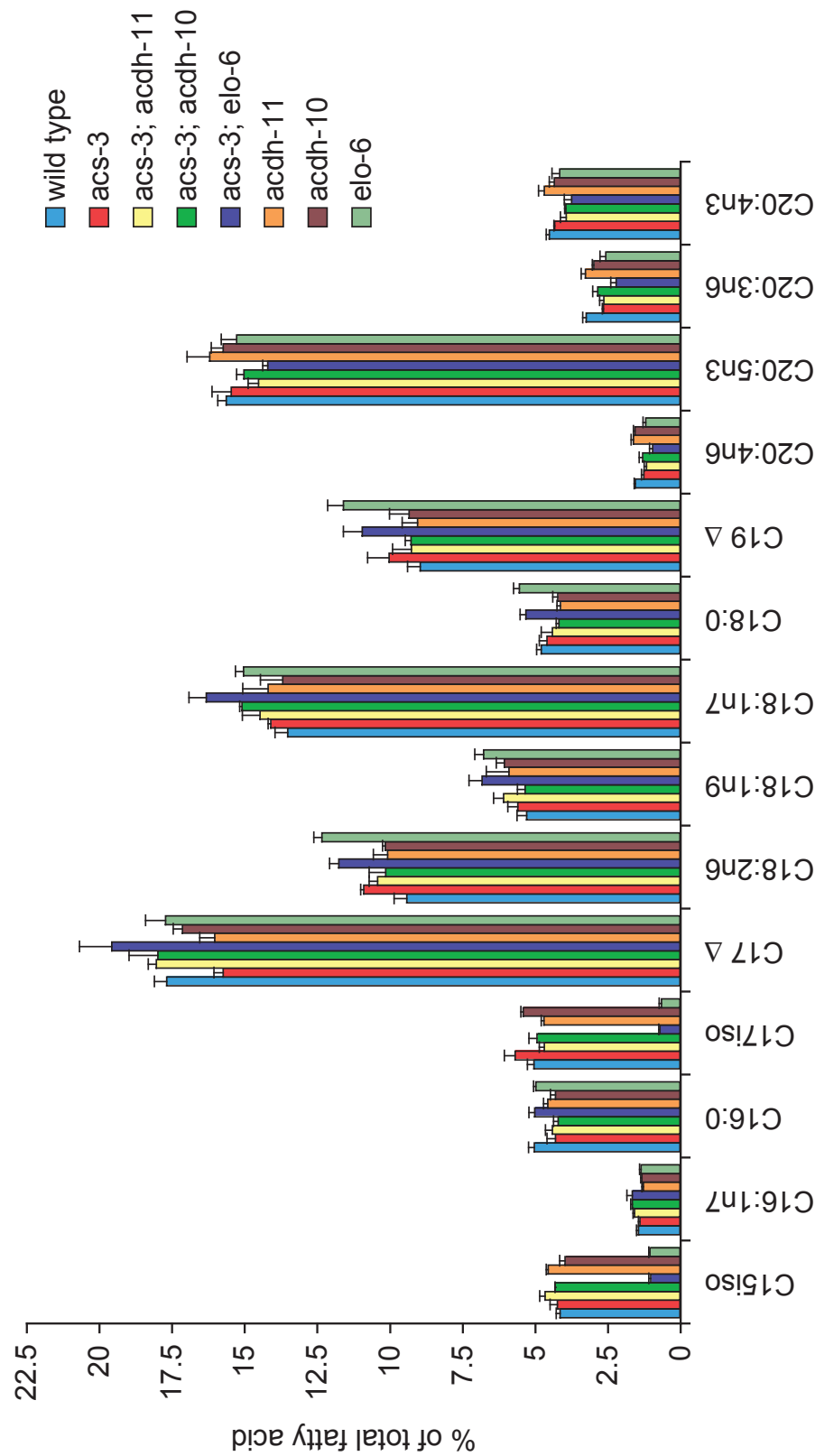
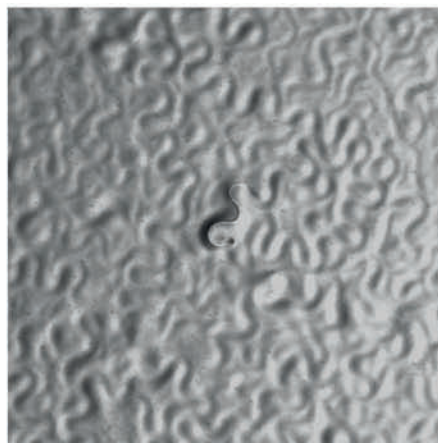


Figure 3-S7



wild type LY



acs-3(ft5) LY



acs-3(ft5); elo-6(ft14) LY



acs-3(ft5); elo-6(ft14) LY
C17iso supplementation



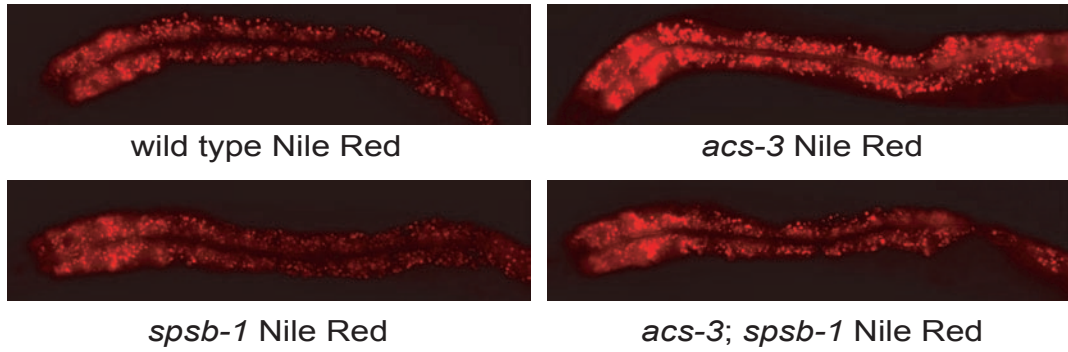
elo-5 RNAi



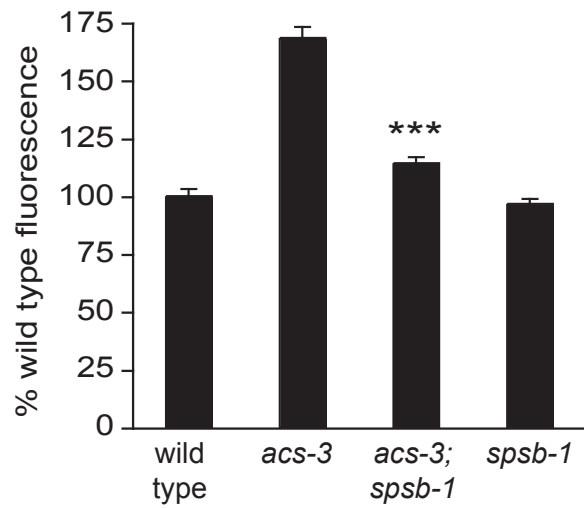
elo-5 RNAi C17iso
supplementation

Figure 3-S8

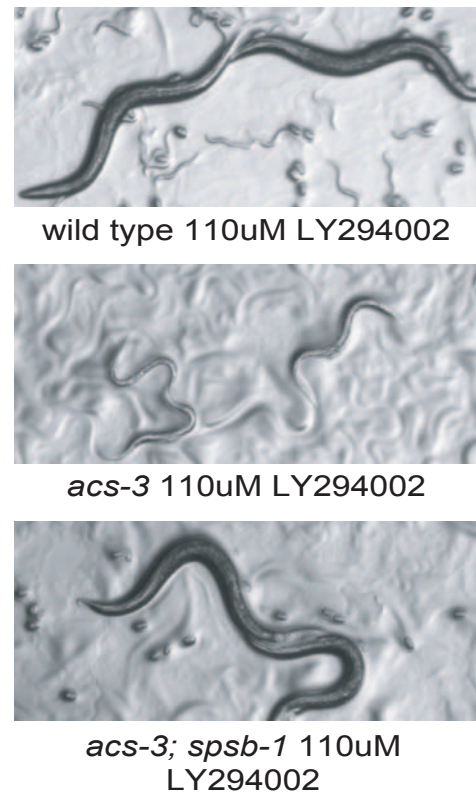
A



B



C



D



Figure 3-S9

acs-3 (ft5) V mapping

Lysate	pkP5076 (V:-17.5)	snp_T10B5 (V:-16.95)	pkP5077 (V:- 15.88)	uCE5-828 (V:- 12.9)	pkP5067 (V:3.3)
122	HET	HET	N2	N2	N2
131	N2	N2	N2	HET	CB
133	N2	N2	N2	HET	HET
137	N2	N2	N2	N2	HET
138	N2	N2	N2	HET	HET
144	HET	HET	N2	N2	N2
165	N2	N2	N2	HET	HET
186	N2	N2	N2	HET	HET
217	HET	N2	N2	N2	N2
271	N2	N2	CB	CB	CB
278	N2	N2	N2	HET	HET

elo-6 (ft14) IV mapping

Recombinant	pkP4034 (0.53)	snp_H06H21[1] (1.36)	pkP4035 (1.43)	snp_ZK354[2] (1.59)	uCE-915 (1.67)	pkP4073 (1.78)
56	N2	N2	N2	N2	HET	HET
118	HET	N2	N2	N2		N2
270	N2	N2	N2	N2	N2	HET
317	HET	N2	N2	N2		N2
335	HET	HET	HET	N2		N2
355	HET	HET	N2	N2		N2
460	HET	HET	HET	HET	N2	N2
486	HET	HET	N2	N2		N2
507	N2	N2	N2	N2	HET	HET
560	HET	N2	N2	N2		N2
567	HET		N2	N2		N2
573	N2	N2	N2	N2	HET	HET
652	HET	N2	N2	N2		N2
674	HET	HET	N2	N2		N2
681	HET	HET	N2	N2		N2
719	HET	HET	N2	N2		N2
801	HET	N2	N2	N2		N2
804	HET	HET	HET	HET	N2	N2

Figure 3-S10

acdH-11 (ft8) III mapping

Recombinant	F42H10[1] (-0.26)	pkP3109 (3.34)	snp_Y45F3[2] (3.67)	snp_Y39A1[9] (3.8)	snp_K11D9[4] (5.42)
2	HET	N2	N2	N2	N2
3	HET	N2	N2	N2	N2
22	HET	N2	N2	N2	N2
38	HET	N2	N2	N2	N2
43	HET	N2	N2	N2	N2
45	N2	N2	N2	N2	HET
73	HET	N2	N2	N2	N2
78	HET	N2	N2	N2	N2
79	N2	N2	N2	HET	HET
89	HET	N2	N2	N2	N2

acdH-10 (ft11) X mapping

Recombinant	pkP6110 (-0.80)	pkP6115 (9.18)	pkP6124 (16.71)	pkP6171 (24.49)
1	HET	N2	N2	N2
3	HET	N2	N2	N2
5	HET	HET	N2	N2
7	HET	HET	HET	N2
8	HET	N2	N2	N2
9	HET	HET	N2	N2
11	HET	HET	HET	N2
14	HET	HET	N2	N2
15	HET	N2	N2	N2
34	HET	HET	HET	N2
38	HET	HET	HET	N2
40	HET	HET	HET	N2
51	HET	HET	HET	N2
52	HET	HET	HET	N2
59	HET	HET	HET	N2
66	HET	HET	HET	N2
71	HET	HET	HET	N2
83	HET	HET	HET	N2
91	HET	HET	HET	N2
93	N2	N2	N2	HET
94	HET	HET	HET	N2

Figure 3-S11

spsb-1 (ft13) II mapping

Recombinant	pkP2112 (13.61)	Y39G8B:11219 (21.76)	K10H10[4] (23.09)	Y46E12BR[1] (24.53)
2	HET	N2	N2	N2
3	HET	HET	N2	N2
4	HET	HET	N2	N2
7	HET	N2	N2	N2
13	HET	N2	N2	N2
15	HET	N2	N2	N2
16	HET	N2	N2	N2
17	HET	N2	N2	N2
18	HET	N2	N2	N2
19	HET	N2	N2	N2
20	CB	N2	N2	N2
21	HET	HET	N2	N2
24	HET	N2	N2	N2
25	HET	HET	N2	N2
26	HET		N2	N2
29	HET	N2	N2	N2
31	HET	N2	N2	N2
33	HET	N2	N2	N2
34	HET	N2	N2	N2
40	HET	N2	N2	N2
41	HET	N2	N2	N2
44	HET	N2	N2	N2
45	HET	N2	N2	N2
47	CB	N2	N2	N2
53	HET	N2	N2	N2
61	CB	N2	N2	N2
62	HET	N2	N2	N2
63	HET	N2	N2	N2
64	HET	N2	N2	N2
68	HET	N2	N2	N2
72	HET	N2	N2	N2
74	HET	N2	N2	N2
75	HET	HET	N2	N2
78	HET		N2	N2
79	HET	N2	N2	N2
80	HET	N2	N2	N2
84	HET	N2	N2	N2
85	HET	N2	N2	N2
86	HET	N2	N2	N2
89	HET	HET	N2	N2
90	HET	N2	N2	N2
91	HET	N2	N2	N2
94	HET	N2	N2	N2
95	HET	N2	N2	N2
96	HET	N2	N2	N2
99	HET	N2	N2	N2
104	HET	HET	N2	N2
105	HET	HET	N2	N2
108	HET	HET	N2	N2
132	HET	HET	N2	N2
136	HET	HET	N2	N2
201	HET	HET	HET	HET
238	HET	HET	N2	N2
243	HET	HET	N2	N2
244	HET	HET	N2	N2
256	CB	CB	N2	N2
264	HET	HET	N2	N2
267	HET	HET	HET	HET
279	HET	HET	N2	N2
281	HET	HET	N2	N2
282	HET	HET	N2	N2
286	HET	HET	N2	N2
290	HET	HET	N2	N2
306	HET	HET	N2	N2
321	HET	HET	N2	N2
328	HET	HET	N2	N2
352	HET	HET	N2	N2
356	HET	HET	N2	N2
360	HET	HET	N2	N2
370	HET	HET	HET	N2
371	HET	HET	N2	N2

Chapter III Appendix

Measurement of Fatty Acyl-CoA species

The function of acyl-CoA synthase genes is to generate fatty acyl-CoA species. Presumably, mutation of *acs-3* leads to disruption of the formation of a subset of these metabolites. Furthermore, we have demonstrated that loss of *acdH-10*, *acdH-11* and *elo-6* can compensate for mutation of *acs-3*, we suspect by causing reduced consumption of the fatty acyl-CoA pool. We hoped that we would be able to identify the pool(s) of fatty acyl-CoAs that is reduced upon mutation of *acs-3*, and also to measure any compensatory changes caused by loss of *acdH-10*, *acdH-11* and *elo-6*. To perform such an analysis, a number of technical obstacles needed to first be overcome. The first challenge in detection of fatty acyl-CoAs is the relatively low abundance of these metabolites. Extraction of these metabolites from tens of thousands of worms generally yielded nanomolar to femtomolar quantities of the various fatty acyl-CoA species. Therefore an extremely sensitive detection method was required. This also presented a second challenge; even small amounts of other contaminants from the extraction could completely mask the signal produced by the species we wished to measure. It was therefore important to develop a method to efficiently separate contaminants from the fatty acyl-CoA species. Finally, our genetics would seem to suggest that fatty acyl-CoAs of certain lengths, rather than the entire pool of all fatty acyl-CoAs, are affected by mutations in *acs-3*, *acdH-10*, *acdH-11* and *elo-6*. Simply measuring the entire combined pool of all fatty-acyl CoAs may not reveal potential changes limited to specific species. Therefore, it was important to be able to separate fatty acyl-CoAs of different chain

lengths (as well potentially as different degrees of saturation) in order to acquire measurements of individual species.

After trying a number of extraction, separation and detection techniques unsuccessfully, we adapted a method described in the plant literature (Larson and Graham, 2001). The major improvement provided by this method was the reaction of extracted fatty acyl-CoAs with chloroacetaldehyde, to produce a fluorescent derivative of CoA which improved the detection signal many fold, while simultaneously reducing the noise in the measurements. The other important aspect of this protocol, was the use of four-mobile-phase HPLC. This allowed for on-column cleaning of the sample, greatly reducing background peaks within the region that fatty acyl-CoA species eluted. These improvements made it possible for us to measure the relative area of more than a dozen peaks, representing different fatty acyl-CoA species. Because in the end we were not able to identify clear changes in either *acs-3(ft5)* mutants, or any of the suppressor mutations on their own, or in combination with *acs-3(ft5)*, we did not attempt to identify the species corresponding to these peaks.

We measured the relative areas of 16 peaks that could be easily and reproducibly identified in our HPLC chromatograms (Figure 3-A1). These peaks represent the most abundant species, but numerous smaller peaks were ignored, and some of these likely represent low abundant species. Only small changes were apparent between wild type animals, and *acs-3* mutants. We had hoped to identify one or a few peaks that were reduced in *acs-3* animals, and increased in *acs-3* with suppressor mutations. No such change was apparent (Figure 3-A2). One possible explanation for our failure to find a change in these metabolites with loss of *acs-3* is suggested by the limited expression

pattern of this gene. The seam cells, vulval cells and head cells which express *acs-3* likely represent a small fraction of the total tissue in an animal. Changes in one or a few species of fatty acyl-CoAs within these tissues may not be apparent in an extract from the entire animal. If one could separate the *acs-3*-expressing cells from the animal, and measure these metabolites specifically in those cells, perhaps a change would be apparent. Such an approach is currently not technically feasible. However, it is also possible that the change is limited to a subcellular region, and that even if *acs-3*-expressing cells could be isolated, subcellular changes would not be detectable.

The one change which was clearly apparent in the HPLC profiles of the mutants tested, was the disappearance of single peak in *elo-6* and *acs-3; elo-6* mutants. Peak 8 was completely gone in these mutants (Figure 3-A2). It is likely that this peak represents C17iso:CoA, as this fatty acid was also missing in Marc Van Gilst's fatty acid profile of these mutants.

Despite the failure of this approach to identify relevant fatty acyl-CoA changes in the backgrounds tested, this method was effective in separating and detecting these metabolites, and may prove useful for future analysis of other pathways.

Extraction and Fluorescent Derivization Protocol (adapted from (Larson and Graham, 2001))

1. Grow up 4000 (or 45,000 L2/L3s, grown for 20 hours) synchronized worms (30-40ul pellet), remove as much liquid as possible and freeze at -80 in a 1.5ml tube
2. Add 200ul of freshly prepared extraction buffer

Extraction Buffer

2ml 2-propanol
2ml pH 7.2 50mM KH₂PO₄
50ul glacial acetic acid
80ul 50 mg/ml BSA

3. Grind tissue using drill with polypropylene pestle for 1-2 minutes until most worms appear broken up
4. To remove lipids and pigments, wash the extract 3 times with 200ul petroleum ether (40-60 degrees C elutant) saturated with 1:1 2-propanol:water. Between washes, separate the phases by low-speed centrifugation (100g)
5. Add 5ul saturated $(\text{NH}_4)_2\text{SO}_4$ and vortex briefly
6. Add 600ul 2:1 methanol:chloroform
7. Vortex the tubes and leave on the bench at RT for 20 minutes
8. Centrifuge at 16,000g for 3 minutes
9. Transfer the supernatant to a new tube, and dry at 40 degrees in a SpeedVac system (2-3 hours)
10. Once dry, reconstitute samples in 55ul of 1X Chloroacetaldehyde derivitizing reagent

Chloroacetaldehyde derivitizing agent

0.15 M sodium citrate

0.5% SDS (w/v)

pH 4.0 with NaOH

0.5 M chloroacetaldehyde (7 Molar stock from sigma)

11. Incubate in HPLC for 20 minutes at 85 degrees C
12. Allow to cool, spin 1 minute at max speed to precipitate undissolved material, pipet 25ul into HPLC sample tube
13. Inject 20ul for run

Quaternary HPLC Protocol (adapted from (Larson and Graham, 2001))

Buffers:

- A) 0.25% (v/v) triethylamine in water
- B) 90% (v/v) acetonitrile in water
- C) 90% (v/v) acetonitrile + 1% (v/v) acetic acid in water
- D) 1% (v/v) acetic acid in water

Column temperature 40° C

Gradient protocol

0 minutes:	10% C	90% D	0.75 ml/min
0-5 minutes	80% C	20% D	0.75 ml/min
5-5.1 minutes	80% A	20% D	0.75 ml/min
5.1-7 minutes	97% A	3% B	0.75 ml/min
7-10 minutes	95% A	5% B	0.75 ml/min
10-10.1 minutes	95% A	5% B	0.5 ml/min
10.1-60 minutes	45% A	55% B	0.5 ml/min
60-62 minutes	100% B		0.5 ml/min
62-62.1 minutes	100% B		0.75 ml/min
62.1-65 minutes	100% B		0.75 ml/min
65-65.1 minutes	10% C	90% D	0.75 ml/min

65.1-70 minutes 10% C 90% D 0.75 ml/min

Approximate volumes required per run:

A: 23 ml

B: 13 ml

C: 3 ml

D: 4 ml

Microarray analysis of *acs-3(ft5)* mutants and *nhr-25* RNAi treated animals

Our model suggests that *acs-3* acts to normally inhibit *nhr-25*, which in turn regulates a number of processes, including intestinal lipid storage, gonad and vulva development and sensitivity to LY294002. NHR-25 is a nuclear hormone receptor, and therefore functions to regulate gene transcription, though non-transcriptional roles for this protein have also been described (Asahina et al., 2006; Silhánková et al., 2005). We wished to determine whether *acs-3* affects transcription of *nhr-25*, in order to better understand this pathway. At the time of these experiments, we did not yet have the *nhr-25(ku217)* allele, so we utilized *nhr-25* RNAi. We compared gene changes in: (1) wild type animals *vs.* in *acs-3(ft5)* mutants, (2) wild type animals fed vector control bacteria *vs.* wild type animals treated *nhr-25* RNAi expressing bacteria, (3) wild type animals *vs.* *acs-3(ft5); elo-6(ft14)* and (4) *acs-3(ft5)* *vs.* *acs-3(ft5); elo-6(ft14)*.

All animals were grown at 20° C to the L4 stage, RNA was extracted, and hybridization to spotted arrays generated by the WashU facility was performed by the Fred Hutchison Cancer Research Center microarray facility. Each comparison was conducted in triplicate. From the data, we generated lists of genes that changed by at least 1.5 fold in all three replicates. From these lists a number of clear relationships among the conditions compared emerged. In both *acs-3(ft5)* mutant animals and wild type animals treated with *nhr-25* RNAi, the expression of many genes was changed

significantly compared to wild type control animals. In addition, many genes that changed in *acs-3(ft5)* mutants also changed in *nhr-25* RNAi treated animals. The surprising finding was that there existed an extremely high overlap in genes upregulated in both *acs-3(ft5)* mutants and *nhr-25* RNAi treated animals, as well as an extremely high overlap in genes downregulated in both conditions. However there was almost no overlap between genes upregulated in one condition and downregulated in the other. It therefore seems that many genes are regulated in the same manner by loss of *acs-3* and loss of *nhr-25*.

Our genetic data indicate that *nhr-25* is inhibited by *acs-3*, while this array data would seem to suggest that *acs-3* is activating *nhr-25*. Though the reason for this apparent contradiction is unknown, we consider a number of possibilities. First, both the array signatures of *acs-3* and *nhr-25* RNAi treated animals are somewhat similar to those reported for animals with cuticular defects. It is possible that disruption of the pathway, either by over-activation or under-activation, leads to disruption of the cuticle, yielding similar expression changes. Another possibility is that these genes have independent roles in regulation of cuticle function, separate from the pathway in which they both function to regulate metabolism and other phenotypes. If this were the case, the expression changes resulting from cuticular damage may swamp out signals from other pathways in which the genes function. Alternatively, it may be the case that *acs-3* regulation of *nhr-25* is complex, and that *acs-3* may activate *nhr-25* transcriptional responses, but inhibit its non-transcriptional functions. Finally, regulation of *nhr-25* by *acs-3* may be context dependent. The relationship may switch based on tissue or developmental stage. Perhaps the presence of other factors determines whether the *acs-3*

signal is activating or inhibiting. To differentiate among these and other possibilities, additional investigation of this relationship will be necessary.

We also examined the effect of *elo-6* mutation on *acs-3* dependant changes. We found clear evidence that loss of *elo-6* suppresses transcriptional changes caused by the *acs-3(ft5)* mutation. This relationship is perhaps not surprising.

Analysis of overlap

Genes downregulated in <i>acs-3</i> mutants	352
Genes downregulated with <i>nhr-25</i> RNAi treatment	636
Expected overlap by chance	11.19
Actual overlap	170
Hypergeometric distribution	1×10^{-163}

Genes upregulated in <i>acs-3</i> mutants	649
Genes upregulated with <i>nhr-25</i> RNAi treatment	1081
Expected overlap by chance	35.07
Actual overlap	376
Hypergeometric distribution	1×10^{-300}

References

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Figure Legends

Figure 3A-1

An HPLC chromatogram is shown. Many peaks are present, 16 of the largest are numbered. The areas of these peaks were measured for each condition, and the relative areas of the peaks are depicted in Figure 3A-2

Figure 3A-2

Relative peak areas for each genotype are shown. No significant differences between wild type and *acs-3(ft5)* animals are apparent. *elo-6(ft14)* animals lack peak 8, likely C17iso.

Tables 3A-1 to 3A-4

Genes changed by at least 1.5-fold in all three replicates

Figure 3-A1

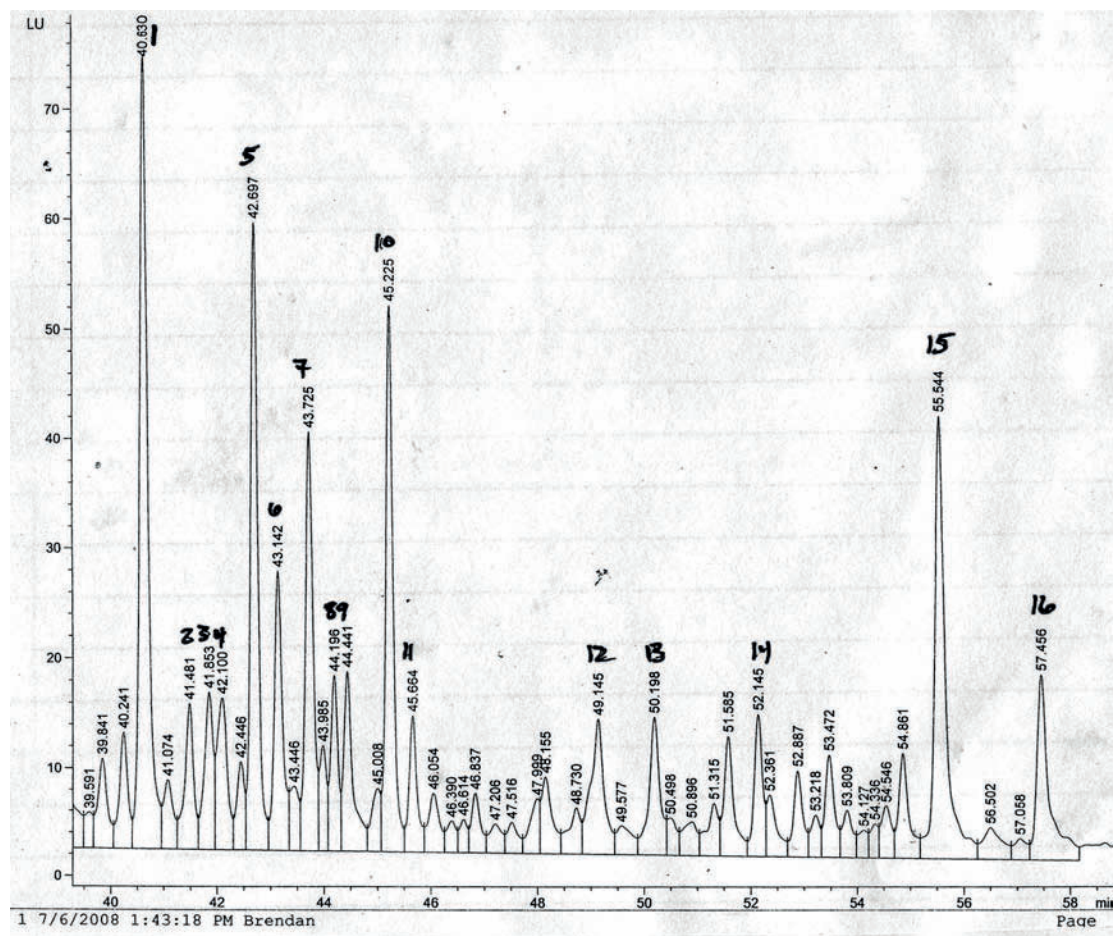


Figure 3-A2

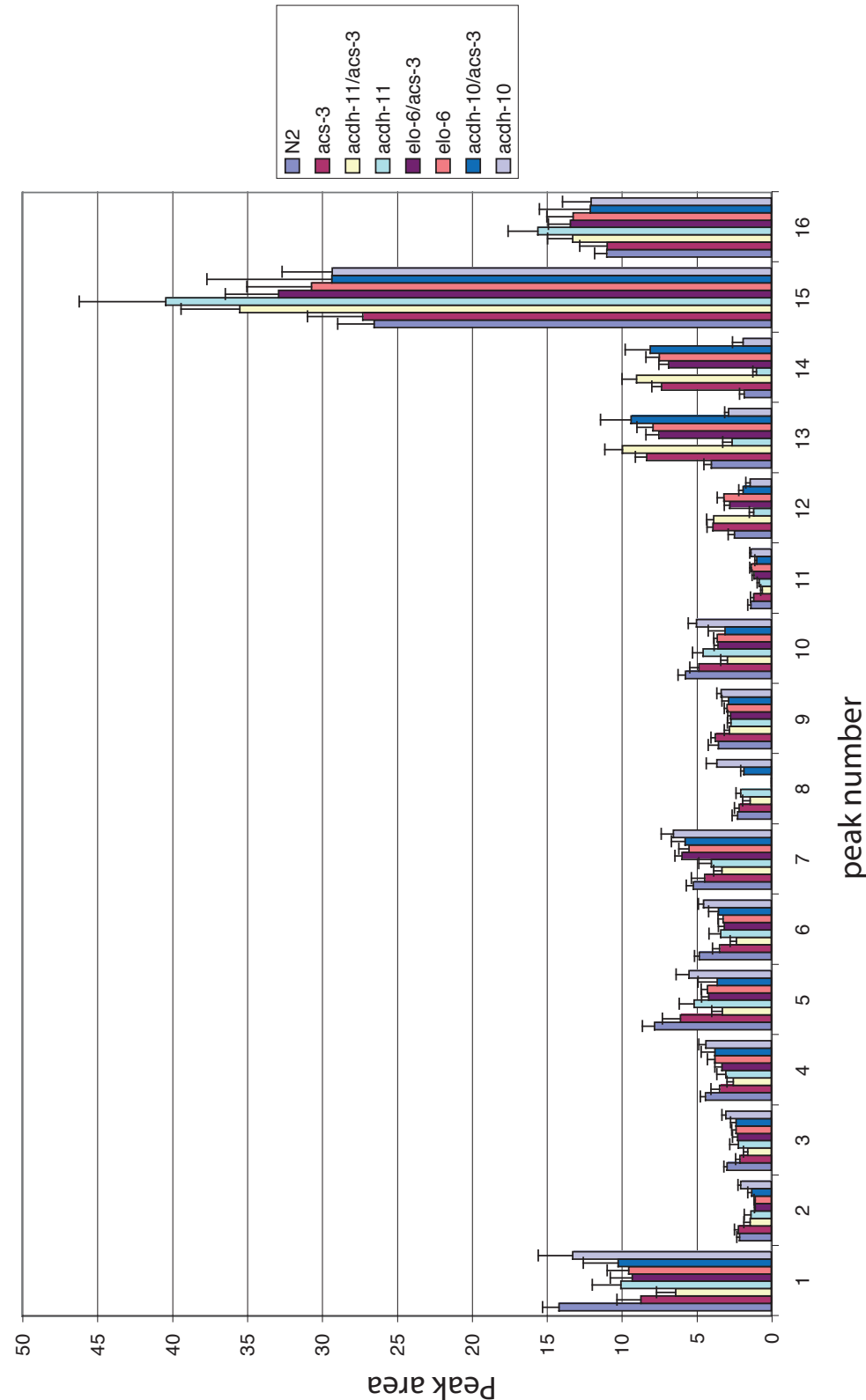


Table 3A-1

Genes upregulated in *acs-3(ft5)* mutant

C. elegans (07/14/09)	Gene Ids	Gene Name	Average log2 change	Std Dev	P value
cea2.i.48192	Y80D3A.7	ptr-22	7.96	7.45	0.20546
cea2.c.37695	R09B5.9	cnc-4	6.01	0.78	0.00551
cea2.d.00017	B0213.17	nlp-34	5.85	1.10	0.01167
cea2.d.03943	F53H2.2	cnc-7	5.47	1.39	0.02071
cea2.c.03774	F48C1.9		5.11	1.58	0.03027
cea2.i.11021	F12A10.1		5.09	1.16	0.01689
cea2.p.11393	F53B6.9	fip-6	4.76	0.65	0.00626
cea2.i.26654	F57H12.3	ora-1	4.65	0.52	0.00411
cea2.p.113075	F15B9.1	far-3	4.55	0.45	0.00321
cea2.p.87872	C42D4.3		4.54	0.81	0.01046
cea2.p.11392	F53B6.8	fipr-26	4.25	0.75	0.01023
cea2.c.33210	B0213.6	nlp-31	4.25	0.32	0.00187
cea2.p.89237	C53B4.8		3.87	0.67	0.00985
cea2.p.32647	C38C6.6	tag-297	3.86	1.04	0.02318
cea2.p.143519	F36G3.1		3.84	1.49	0.04658
cea2.i.30269	Y46C8AL.2	clec-174	3.81	0.54	0.00658
cea2.i.30418	Y51H4A.5		3.78	1.59	0.05423
cea2.d.00018	B0213.5	nlp-30	3.75	0.83	0.01590
cea2.i.53522	F35B3.4		3.74	0.11	0.00030
cea2.d.16965	C45B2.8		3.73	0.81	0.01531
cea2.c.38593	T19B10.2		3.70	0.90	0.01913
cea2.p.88062	C45G7.2	ilys-2	3.57	0.46	0.00557
cea2.p.117672	F52F10.2		3.56	0.81	0.01683
cea2.p.145574	F47E1.2		3.46	0.53	0.00764
cea2.p.147751	F57C12.1	nas-38	3.32	0.94	0.02587
cea2.p.148241	F59F4.1		3.30	0.75	0.01654
cea2.i.47134	Y43F8C.1	nlp-25	3.28	1.10	0.03524
cea2.c.32759	ZC168.5	grl-21	3.27	1.34	0.05141
cea2.d.01404	C45B2.2		3.26	0.66	0.01334
cea2.p.33276	C50D2.1		3.23	0.93	0.02641
cea2.p.101026	Y38C1BA.3	col-109	3.21	1.01	0.03146
cea2.d.41508	W08E12.3		3.18	0.61	0.01200
cea2.p.121319	K11D12.4	cpt-4	3.15	0.98	0.03095
cea2.p.131101	ZC443.5	ugt-18	3.04	1.69	0.08938
cea2.p.24495	Y95B8A.2		3.03	0.51	0.00928
cea2.c.36886	F59E11.7		3.01	0.37	0.00496
cea2.p.53061	ZK84.1		3.01	1.38	0.06326
cea2.i.41492	K09D9.1		3.01	0.47	0.00789
cea2.p.115349	F28G4.1	cyp-37B1	2.96	1.25	0.05485
cea2.p.22926	Y54E10BL.2	col-48	2.95	1.46	0.07249
cea2.d.41903	Y105C5A.4	abu-5	2.95	1.40	0.06732
cea2.c.25574	ZK1010.7	col-97	2.94	1.17	0.04851
cea2.d.08484	B0213.3	nlp-28	2.93	1.50	0.07756
cea2.p.53984	ZK1067.7	pqn-95	2.91	1.51	0.07880
cea2.p.139449	F09B9.1	oac-14	2.91	0.68	0.01762
cea2.i.11035	F12A10.7		2.89	1.33	0.06366
cea2.i.37297	F21F8.2		2.88	1.31	0.06276
cea2.c.15098	Y38E10A.15		2.86	0.25	0.00245
cea2.i.18528	C02F5.11	tsp-2	2.85	0.43	0.00760
cea2.c.36514	F53F1.5		2.85	1.25	0.05883
cea2.p.88082	C46A5.3	col-14	2.85	1.62	0.09257

cea2.c.49413	R12E2.7		2.83	0.85	0.02861
cea2.i.38512	F36H9.2		2.82	0.52	0.01126
cea2.d.08299	AC3.3	abu-1	2.81	1.16	0.05224
cea2.d.01923	E03H4.10	clec-17	2.81	2.05	0.14178
cea2.c.29283	F58F9.7		2.78	0.37	0.00569
cea2.d.08308	AC3.3	abu-1	2.78	1.05	0.04410
cea2.c.44495	F35A5.3	abu-10	2.78	0.74	0.02295
cea2.p.123158	T02E9.2	grl-7	2.76	1.71	0.10817
cea2.p.102003	Y46C8AL.5	clec-72	2.75	0.37	0.00596
cea2.d.47041	Y73F8A.9	pqn-91	2.70	1.72	0.11270
cea2.p.43895	R03C1.2		2.70	0.66	0.01954
cea2.i.19343	F22B7.4	fip-1	2.70	0.39	0.00676
cea2.p.109736	C26F1.2	cyp-32A1	2.68	0.90	0.03580
cea2.c.35219	F15B9.8		2.68	1.23	0.06331
cea2.p.112183	F08F3.7	cyp-14A5	2.67	0.72	0.02329
cea2.d.47046	Y73F8A.9	pqn-91	2.67	1.61	0.10298
cea2.d.03076	F35C5.10	nsps-11	2.66	0.39	0.00724
cea2.c.37693	R09B5.8	cnc-3	2.65	0.33	0.00524
cea2.p.106721	B0024.4		2.63	0.50	0.01178
cea2.p.21595	Y47H10A.5		2.62	0.61	0.01743
cea2.p.157687	ZK662.2		2.61	1.30	0.07360
cea2.3.30545	Y73F8A.8	pqn-90	2.61	1.13	0.05720
cea2.c.15603	Y57A10C.6		2.60	1.05	0.05032
cea2.c.11224	F13D12.3		2.59	0.19	0.00173
cea2.p.110332	C35A5.2	ugt-33	2.58	0.70	0.02390
cea2.i.58840	F09F7.8	nsps-12	2.56	0.49	0.01193
cea2.p.37624	F26G1.5		2.54	0.79	0.03036
cea2.d.65625	R12E2.15		2.54	1.36	0.08425
cea2.i.26215	F45E4.5		2.52	0.92	0.04152
cea2.p.119310	F58E6.1a		2.51	1.26	0.07400
cea2.c.36290	F41F3.3		2.51	1.51	0.10185
cea2.i.42032	M03F8.1		2.50	0.86	0.03690
cea2.d.08100	Y41E3.2	dpy-4	2.49	1.65	0.12098
cea2.p.89456	D2096.6		2.47	1.45	0.09821
cea2.c.13679	R05H10.1		2.47	1.37	0.08975
cea2.p.123901	T06E4.8		2.46	1.45	0.09924
cea2.p.28829	C01G6.7		2.45	0.75	0.02997
cea2.c.33505	C02E7.6		2.44	1.25	0.07737
cea2.d.16966	C45G7.3	ilys-3	2.44	1.08	0.06007
cea2.i.21452	Y119D3B.9	fbxa-21	2.43	1.25	0.07764
cea2.d.48550	ZK180.5b		2.43	1.25	0.07862
cea2.d.19988	F11G11.12	col-73	2.42	1.43	0.09890
cea2.p.41525	F59B10.3		2.42	0.90	0.04323
cea2.c.20582	H10E21.4		2.41	1.03	0.05537
cea2.i.13348	F58E1.6	nhx-6	2.40	1.53	0.11278
cea2.i.13260	F58A6.1		2.40	0.28	0.00437
cea2.c.16485	C02F5.8	tsp-1	2.39	0.77	0.03286
cea2.i.28886	W08E12.2		2.39	1.02	0.05594
cea2.i.38539	F36H9.5		2.39	0.11	0.00076
cea2.d.22581	F29D10.2		2.38	1.16	0.07126
cea2.c.33506	C02E7.7		2.38	1.07	0.06113
cea2.p.158399	R12E2.14		2.37	1.02	0.05630
cea2.c.17582	C29E4.1	col-90	2.37	0.72	0.02907
cea2.c.13879	T01D1.6	abu-11	2.36	1.00	0.05477
cea2.p.38017	F29C12.1	pqn-32	2.36	1.44	0.10512
cea2.i.07343	Y95B8A.1	nas-30	2.36	1.49	0.11137
cea2.p.158110	T06E4.9		2.35	0.86	0.04209
cea2.i.46562	Y37H2A.11		2.35	0.53	0.01623
cea2.p.34685	E01G4.6		2.35	1.66	0.13475

cea2.p.67860	K04C2.5		2.34	0.85	0.04135
cea2.c.31899	Y43C5A.3		2.33	1.86	0.16174
cea2.p.39714	F44F4.4	ptr-8	2.33	0.28	0.00473
cea2.p.56525	C06E8.5		2.32	0.39	0.00937
cea2.i.39600	F52E1.8		2.32	1.19	0.07716
cea2.p.80080	Y75B8A.4		2.31	0.28	0.00491
cea2.p.77723	Y47D3B.6		2.31	1.24	0.08354
cea2.p.128465	Y43F8B.3		2.31	1.16	0.07457
cea2.p.32490	C34F11.8		2.30	0.91	0.04824
cea2.p.55655	BE10.2		2.30	0.85	0.04292
cea2.p.32628	C38C6.3		2.30	0.29	0.00530
cea2.3.20798	M162.5		2.30	1.37	0.10105
cea2.d.45414	Y54G11A.5b		2.29	0.85	0.04254
cea2.i.51977	C54D2.1		2.29	0.53	0.01727
cea2.p.68358	K08E3.1	tyr-2	2.29	0.39	0.00948
cea2.p.14381	K08C9.4	col-65	2.28	1.44	0.11132
cea2.p.111104	C49G7.10		2.28	0.30	0.00583
cea2.i.43867	T06E4.10		2.28	1.13	0.07306
cea2.i.21642	Y37B11A.1	cutl-23	2.28	0.30	0.00557
cea2.p.75397	W05G11.3	col-88	2.27	0.88	0.04690
cea2.i.42355	R07B5.3	sru-40	2.26	1.65	0.14063
cea2.p.113267	F16B4.7		2.25	0.50	0.01579
cea2.p.80054	Y75B8A.3		2.24	0.30	0.00607
cea2.i.22476	Y75B8A.20	grl-15	2.24	0.90	0.05044
cea2.p.77872	Y48A6B.9		2.22	0.29	0.00572
cea2.p.15623	R11A5.7		2.22	0.86	0.04649
cea2.c.10341	C30B5.6		2.21	0.98	0.06012
cea2.p.107640	C03G6.3	srj-14	2.20	1.19	0.08513
cea2.i.08538	C01G6.9		2.19	1.12	0.07704
cea2.i.18053	ZK666.6	clec-60	2.19	1.06	0.06997
cea2.i.47618	Y51A2B.1		2.18	1.28	0.09866
cea2.3.30579	Y76B12C.5		2.18	0.41	0.01164
cea2.p.20022	Y18H1A.9		2.17	0.94	0.05764
cea2.p.38384	F33A8.7		2.16	1.29	0.10090
cea2.3.29824	Y58A7A.4		2.16	0.34	0.00827
cea2.p.129486	Y58A7A.3		2.16	0.41	0.01161
cea2.c.28966	F53B2.8		2.15	0.34	0.00824
cea2.i.06712	Y65B4BL.6		2.14	0.85	0.04914
cea2.c.38944	T28H10.3		2.13	0.31	0.00714
cea2.p.135278	C29F7.2		2.13	0.40	0.01177
cea2.p.115750	F32D8.7		2.12	0.60	0.02579
cea2.c.05339	R11A5.3		2.11	0.57	0.02329
cea2.p.130005	Y75B7AR.1		2.10	1.45	0.12810
cea2.c.03270	F33D11.8		2.10	0.68	0.03344
cea2.p.105706	ZK180.6		2.09	1.39	0.12124
cea2.c.39795	Y47D7A.5	grl-5	2.09	1.30	0.10793
cea2.p.127661	Y32G9B.1		2.09	0.92	0.05946
cea2.i.58329	ZC373.6	dao-4	2.09	1.08	0.07910
cea2.c.07850	Y65B4BR.6	grl-16	2.09	1.06	0.07611
cea2.d.00092	B0238.12		2.09	0.98	0.06651
cea2.p.46239	T06D8.3		2.09	1.29	0.10758
cea2.3.03031	C14A6.2		2.08	1.22	0.09781
cea2.p.127447	Y22F5A.6	lys-3	2.07	1.01	0.07119
cea2.d.09988	C01G12.6	nspb-10	2.06	0.44	0.01474
cea2.p.72574	T05D4.4	osm-7	2.06	0.09	0.00068
cea2.d.02199	F11G11.10	col-17	2.06	1.37	0.12190
cea2.3.07413	D1009.1b		2.05	1.09	0.08312
cea2.p.150597	M03F4.6		2.05	0.61	0.02864
cea2.p.128967	Y47D7A.13		2.05	1.17	0.09393

cea2.c.08961	B0491.2	sqt-1	2.04	1.56	0.15140
cea2.p.124789	T16G1.4		2.03	0.98	0.07010
cea2.c.10738	E04F6.3	maoc-1	2.02	0.31	0.00781
cea2.3.19098	K07B1.8		2.02	0.57	0.02590
cea2.i.29324	Y11D7A.5		2.02	1.15	0.09358
cea2.3.07384	D1009.1a		2.01	1.15	0.09342
cea2.p.45954	T05C12.10	qua-1	1.99	1.33	0.12318
cea2.i.40574	F59E11.6		1.98	0.40	0.01363
cea2.p.91466	F29B9.9	col-111	1.98	0.84	0.05539
cea2.i.40519	F59E11.12	nhr-144	1.96	1.26	0.11412
cea2.p.110908	C45B11.3	dhs-18	1.96	0.51	0.02175
cea2.3.24071	T16G1.1	pqn-67	1.96	0.76	0.04634
cea2.p.158153	C13B9.1		1.96	1.54	0.15889
cea2.p.33309	C50D2.6		1.96	1.37	0.13136
cea2.c.47062	M03A8.1	dhs-28	1.95	0.71	0.04121
cea2.i.44641	T16G1.6		1.95	0.36	0.01100
cea2.i.11975	F35C5.7	clec-64	1.94	0.50	0.02140
cea2.p.132982	C06G1.1		1.93	1.11	0.09377
cea2.i.29352	Y11D7A.9		1.93	1.15	0.10040
cea2.p.131164	ZC455.10	fkf-4	1.93	0.71	0.04254
cea2.p.158093	F48G7.5		1.92	0.68	0.03923
cea2.d.38988	T16G1.7		1.92	0.23	0.00479
cea2.i.52437	F08F1.6	spp-13	1.92	1.33	0.12954
cea2.p.113124	F15B9.6		1.91	0.15	0.00207
cea2.c.16346	B0412.4	rps-29	1.91	2.08	0.25369
cea2.d.49145	ZK402.3		1.89	0.13	0.00163
cea2.p.32802	C41D7.2	ptr-3	1.89	0.42	0.01589
cea2.d.08488	B0222.6	col-144	1.89	0.52	0.02440
cea2.p.98912	T21D12.7		1.88	0.67	0.03943
cea2.i.36402	F13A2.6		1.88	0.20	0.00380
cea2.c.50255	Y48G8AL.12		1.88	1.08	0.09445
cea2.i.36844	F16B4.9	nhr-178	1.88	0.44	0.01791
cea2.i.16072	W01D2.4	srt-11	1.88	0.33	0.00997
cea2.c.50239	F20B6.9		1.87	0.64	0.03660
cea2.p.134065	C15H9.1	nnt-1	1.87	0.13	0.00160
cea2.d.08492	B0222.7	col-145	1.86	0.95	0.07705
cea2.d.45431	Y54G11A.6	ctl-1	1.86	0.78	0.05348
cea2.p.127700	Y38A10A.2		1.86	0.68	0.04208
cea2.p.70959	R13A5.3	ttr-32	1.86	1.22	0.11830
cea2.c.32957	ZK550.6		1.85	0.55	0.02792
cea2.c.39838	Y51A2D.11	ttr-26	1.84	0.47	0.02114
cea2.i.49548	B0310.6		1.84	0.24	0.00548
cea2.d.08490	B0222.7	col-145	1.84	0.89	0.06931
cea2.p.33627	C54A12.1	ptr-6	1.84	0.35	0.01167
cea2.p.33320	C50D2.7		1.84	0.12	0.00131
cea2.i.04332	T06G6.6		1.84	0.44	0.01841
cea2.d.46470	Y69H2.3d		1.83	0.51	0.02475
cea2.p.127324	W08G11.1		1.83	0.82	0.06023
cea2.c.34876	C54F6.14	ftn-1	1.83	0.65	0.04025
cea2.c.38631	T19C4.7	nlp-33	1.82	0.29	0.00837
cea2.p.111517	C54F6.5		1.81	0.39	0.01515
cea2.f.08178	Y54G11A.5a		1.81	1.02	0.09193
cea2.p.113487	F17C11.11		1.81	0.89	0.07151
cea2.d.24690	F39D8.1b		1.81	0.99	0.08739
cea2.c.44881	F41E7.4	fip-5	1.81	1.22	0.12447
cea2.i.22348	Y71H2AM.16		1.80	0.96	0.08262
cea2.i.16857	Y46D2A.1		1.80	0.82	0.06295
cea2.i.52503	F09F9.2		1.80	0.09	0.00075
cea2.c.08781	ZK909.3		1.80	0.88	0.07149

cea2.d.24658	F39D8.1a		1.79	0.77	0.05656
cea2.p.33295	C50D2.2		1.79	0.72	0.04922
cea2.c.35197	F10D2.8		1.79	0.33	0.01149
cea2.d.38977	T16G1.7		1.78	0.48	0.02327
cea2.p.35493	F07H5.8		1.77	0.38	0.01509
cea2.c.41347	C14F11.6		1.77	1.05	0.10080
cea2.c.36356	F45D3.4		1.76	0.10	0.00105
cea2.d.07845	F09B12.1b		1.76	1.19	0.12384
cea2.3.19089	K07B1.8		1.75	0.81	0.06395
cea2.i.52887	F18G5.2	pes-8	1.75	0.83	0.06766
cea2.i.07840	B0034.1		1.75	0.23	0.00567
cea2.c.18644	F09G8.6	col-91	1.75	0.82	0.06647
cea2.d.10899	C05E7.1a		1.74	0.56	0.03315
cea2.d.24202	F36H5.2b		1.74	1.37	0.15797
cea2.p.38353	F32A5.6	prx-13	1.74	0.28	0.00864
cea2.p.148515	H13N06.2		1.74	0.16	0.00269
cea2.p.114207	F21C10.9		1.73	0.71	0.05173
cea2.p.126421	W01F3.3	mlt-11	1.73	1.36	0.15882
cea2.p.95467	K08C7.2	fmo-1	1.73	0.64	0.04264
cea2.p.121451	K11G9.5		1.73	0.52	0.02847
cea2.c.25910	B0564.3		1.72	0.29	0.00920
cea2.p.153198	T05A10.3	ttr-14	1.71	0.56	0.03353
cea2.c.45618	F53A9.8		1.71	0.29	0.00919
cea2.i.25633	F28E10.2		1.70	0.08	0.00072
cea2.d.24196	F36H5.2b		1.70	0.45	0.02276
cea2.i.32048	B0348.2		1.70	1.43	0.17461
cea2.p.119593	F59A7.2		1.69	0.78	0.06417
cea2.i.27476	M01H9.1		1.69	0.78	0.06429
cea2.i.58548	ZK377.1	wrt-6	1.69	1.04	0.10589
cea2.p.148821	H42K12.3		1.69	1.36	0.16478
cea2.c.41917	C34E7.4		1.69	0.57	0.03582
cea2.c.31535	W03F8.6		1.69	0.66	0.04732
cea2.p.151418	R07B1.2	lec-7	1.68	0.74	0.05833
cea2.i.41185	K06A4.4		1.68	0.89	0.08272
cea2.p.104136	Y73B6BL.1		1.67	0.75	0.06040
cea2.i.18119	ZK892.2	nlt-1	1.66	0.36	0.01504
cea2.p.119579	F59A1.10		1.65	0.59	0.03967
cea2.c.36452	F47C10.2	btb-21	1.65	0.92	0.08972
cea2.p.116987	F44E7.2		1.65	0.39	0.01842
cea2.c.28931	F49F1.5		1.64	0.36	0.01532
cea2.i.49490	B0272.4		1.64	0.12	0.00181
cea2.i.52136	D2021.4		1.63	0.84	0.07785
cea2.i.39273	F46B6.8		1.63	0.47	0.02706
cea2.i.09370	C18H9.5		1.63	0.13	0.00197
cea2.p.85215	C04C3.5	dyf-3	1.63	0.23	0.00643
cea2.i.22790	ZK1010.4		1.63	0.10	0.00127
cea2.c.31340	T23F6.1		1.62	0.61	0.04392
cea2.i.21483	Y22D7AL.14		1.62	0.07	0.00068
cea2.3.25979	W03F8.2		1.61	1.07	0.11962
cea2.d.10909	C05E7.1b		1.60	0.53	0.03488
cea2.p.44692	R07G3.2	lips-17	1.60	0.22	0.00631
cea2.d.41218	W06B3.2b		1.60	0.44	0.02468
cea2.p.34001	D1069.2	cpn-2	1.60	0.89	0.08982
cea2.i.34104	C33G8.12	nhr-163	1.60	0.38	0.01842
cea2.i.41984	M02H5.4	nhr-202	1.60	1.06	0.12166
cea2.c.41613	C18A11.1		1.59	0.46	0.02697
cea2.i.16553	Y17G7B.8		1.58	0.79	0.07376
cea2.d.47084	Y74E4A.1a		1.57	0.87	0.08855
cea2.d.10889	C05E4.9b		1.57	0.84	0.08344

cea2.i.14934	T05C1.1		1.57	0.36	0.01682
cea2.d.11026	C06B8.2b		1.56	0.48	0.03051
cea2.i.58944	R52.3	math-35	1.56	0.70	0.06039
cea2.i.30353	Y4C6B.2		1.56	0.77	0.07316
cea2.i.50371	C11G10.1		1.55	0.43	0.02447
cea2.p.126569	W03F9.4		1.54	0.22	0.00656
cea2.i.40394	F59A7.1	clec-206	1.54	0.35	0.01721
cea2.p.158055	C06B8.2a		1.52	0.36	0.01814
cea2.i.00636	C30F12.6		1.52	0.14	0.00283
cea2.i.36420	F13A7.11		1.51	0.07	0.00081
cea2.p.138171	C54D2.4	sul-3	1.51	0.89	0.09861
cea2.c.09664	C07E3.3		1.51	0.47	0.03129
cea2.p.94728	H06H21.10	tat-2	1.50	0.35	0.01762
cea2.p.61872	F17C8.2	col-89	1.50	0.67	0.06115
cea2.i.43884	T06E4.6	col-146	1.50	0.89	0.09972
cea2.c.34597	C50B8.4		1.49	0.39	0.02168
cea2.p.121616	M03E7.2		1.49	0.39	0.02204
cea2.3.23199	T08B6.5		1.49	1.12	0.14723
cea2.p.40097	F47F6.8		1.49	0.81	0.08536
cea2.c.12179	F41C3.2		1.49	0.09	0.00131
cea2.i.41972	M02H5.3	nhr-201	1.49	0.22	0.00717
cea2.p.125946	T26H2.9	nhr-79	1.49	0.20	0.00583
cea2.c.38150	T05B4.3	phat-4	1.49	0.41	0.02421
cea2.p.72748	T07A5.3		1.48	0.71	0.06861
cea2.p.102019	Y46C8AR.1	clec-76	1.48	0.35	0.01827
cea2.p.122633	R13H4.3		1.48	0.42	0.02573
cea2.d.49742	ZK666.2		1.48	0.66	0.05989
cea2.i.22663	Y82E9BR.4		1.48	0.27	0.01078
cea2.p.55680	C02C2.1	tyr-1	1.48	0.18	0.00498
cea2.c.31425	T28C6.2		1.48	0.58	0.04780
cea2.c.12153	F38A3.2	ram-2	1.48	0.57	0.04614
cea2.p.157339	ZK154.1		1.48	0.57	0.04583
cea2.i.19219	E02H9.4		1.47	0.55	0.04352
cea2.p.94538	F58G6.6	del-2	1.47	0.82	0.09006
cea2.p.129201	Y51A2D.4	hmit-1.1	1.47	1.24	0.17738
cea2.p.137783	C52B9.2		1.47	0.60	0.05073
cea2.p.119636	F59B1.8		1.46	0.19	0.00579
cea2.i.26874	H04M03.2	nspb-6	1.46	0.24	0.00923
cea2.i.12236	F40H3.3		1.46	0.30	0.01408
cea2.i.19319	F10F2.7	clec-151	1.46	1.32	0.19636
cea2.p.12528	F56C11.6		1.45	0.26	0.01063
cea2.3.15198	F48G7.12		1.45	0.42	0.02667
cea2.c.41840	C26B9.3		1.45	0.78	0.08485
cea2.p.155792	W04G3.3	lpr-4	1.44	0.95	0.11859
cea2.p.121296	K11D12.3	srr-4	1.44	0.34	0.01750
cea2.i.08705	C07D10.4	nas-7	1.44	0.28	0.01258
cea2.c.39182	W06D12.3	fat-5	1.44	0.57	0.04867
cea2.i.58018	Y41G9A.5		1.44	0.67	0.06576
cea2.d.07740	C05E4.9a		1.43	0.51	0.03976
cea2.p.139516	F09E10.7		1.43	0.56	0.04711
cea2.p.124101	T07F10.4	bus-19	1.43	0.68	0.06766
cea2.i.35750	EGAP9.2	fut-2	1.42	0.47	0.03459
cea2.d.10890	C05E4.9b		1.41	0.42	0.02779
cea2.p.39987	F46C5.3	nas-25	1.41	0.25	0.01045
cea2.d.46965	Y73C8B.1		1.41	0.30	0.01525
cea2.d.05783	T21C12.1a		1.41	0.58	0.05179
cea2.c.26820	C39E9.8		1.40	0.27	0.01174
cea2.i.07227	Y87G2A.14	ndx-8	1.40	0.37	0.02256
cea2.p.52983	ZK20.1		1.40	0.11	0.00216

cea2.p.147112	F54F7.3		1.39	0.74	0.08189
cea2.i.56311	R04B3.3		1.39	0.41	0.02822
cea2.i.44504	T11F9.6	nas-22	1.39	0.54	0.04710
cea2.p.77837	Y48A6B.2		1.38	0.66	0.06749
cea2.p.121002	K09H11.1		1.38	0.20	0.00689
cea2.d.17554	C52A10.2		1.38	0.18	0.00553
cea2.d.21380	F20B6.6		1.38	0.56	0.05017
cea2.i.35082	C53A3.2		1.38	0.32	0.01692
cea2.i.42412	R07B7.15	nhr-208	1.38	0.65	0.06658
cea2.d.16632	C44C10.1	col-180	1.38	0.69	0.07337
cea2.p.146532	F52E4.6	wrt-2	1.37	0.61	0.06028
cea2.i.14710	R11F4.3		1.37	0.34	0.02023
cea2.d.08130	Y55F3AM.3c		1.37	0.55	0.05013
cea2.d.33151	K09H11.7		1.37	0.68	0.07247
cea2.p.96611	R07C12.2		1.37	0.31	0.01674
cea2.p.84024	B0218.2		1.37	0.21	0.00799
cea2.p.02203	C17H1.7		1.36	0.58	0.05580
cea2.i.53060	F21E9.3	ttr-37	1.36	0.32	0.01755
cea2.c.47604	T08A9.2	ttr-30	1.35	0.66	0.07108
cea2.i.41577	K09H11.6		1.35	0.98	0.13876
cea2.i.28763	W02A2.5		1.35	0.25	0.01102
cea2.c.33673	C06C6.4	nhr-63	1.35	0.29	0.01526
cea2.d.08183	T12B5.8	fbxa-59	1.34	0.36	0.02263
cea2.p.130516	Y113G7B.14		1.34	0.88	0.11895
cea2.d.05140	T01C4.2a		1.34	0.40	0.02799
cea2.p.106691	AC3.7	ugt-1	1.34	0.36	0.02342
cea2.d.32660	K07C6.14		1.34	0.44	0.03459
cea2.i.33927	C29F3.3		1.33	0.57	0.05570
cea2.3.00423	B0238.13		1.33	0.21	0.00825
cea2.p.107932	C06B3.6		1.33	0.35	0.02225
cea2.p.50120	Y46G5A.11		1.33	0.60	0.06267
cea2.d.06332	W06B3.2a		1.32	0.48	0.04174
cea2.c.48611	Y102A11A.5		1.32	0.83	0.10930
cea2.i.01943	F25H5.8		1.32	0.25	0.01138
cea2.i.25168	F08G5.6		1.32	0.59	0.06018
cea2.p.102693	Y57G11A.1	tag-273	1.32	0.28	0.01511
cea2.p.91665	F32E10.3	clec-180	1.32	0.68	0.07867
cea2.d.30166	F58B3.1	lys-4	1.31	0.23	0.00964
cea2.i.28654	T28C6.1	grsp-2	1.31	0.43	0.03348
cea2.p.54545	B0280.7		1.31	0.30	0.01666
cea2.p.40149	F48A11.1	chs-2	1.31	0.31	0.01806
cea2.d.14673	C28G1.6		1.31	0.32	0.01960
cea2.p.79019	Y55B1BL.1		1.31	0.06	0.00069
cea2.c.21157	K02F3.4	zip-2	1.31	0.29	0.01625
cea2.p.121611	M02H5.6	nhr-98	1.30	0.47	0.04142
cea2.i.41994	M02H5.5	nhr-203	1.30	0.49	0.04339
cea2.p.127610	Y32F6B.1		1.30	0.22	0.00952
cea2.p.07559	F21F3.3		1.30	0.48	0.04317
cea2.c.32848	ZC416.8b		1.29	0.64	0.07379
cea2.p.112063	F02D8.4		1.29	0.16	0.00477
cea2.d.17555	C52A10.2		1.29	0.31	0.01834
cea2.i.21804	Y41C4A.11		1.29	0.51	0.04809
cea2.p.158073	F15H10.1	col-12	1.29	0.49	0.04507
cea2.p.109111	C16D9.4		1.29	0.33	0.02071
cea2.p.26343	ZK270.1	ptr-23	1.29	0.28	0.01493
cea2.p.107338	B0507.1		1.28	0.33	0.02133
cea2.p.23394	Y71F9AL.4		1.28	0.07	0.00108
cea2.p.155464	T27A10.6		1.28	0.50	0.04696
cea2.c.26266	C08F11.13		1.27	0.19	0.00707

cea2.d.20888	F15H10.2	col-13	1.27	0.39	0.03054
cea2.p.129971	Y73C8B.3		1.26	0.34	0.02307
cea2.p.04877	C45E1.4		1.26	0.45	0.03944
cea2.i.11941	F35C11.6		1.26	0.16	0.00541
cea2.p.36472	F14E5.5	lips-10	1.26	0.59	0.06631
cea2.p.85301	C05C12.4		1.26	0.33	0.02265
cea2.i.06838	Y6B3B.10	lagr-1	1.25	0.55	0.05780
cea2.3.29820	Y58A7A.4		1.25	0.31	0.02033
cea2.p.98695	T14A8.2		1.25	0.08	0.00145
cea2.c.12165	F40F8.7	pqm-1	1.25	0.14	0.00390
cea2.p.125294	T20B3.1		1.25	0.15	0.00470
cea2.i.04298	T05E8.2	hil-8	1.25	0.86	0.12959
cea2.i.56831	T04F8.4	cutl-11	1.25	0.74	0.10052
cea2.p.78870	Y54F10AM.6		1.25	0.45	0.04099
cea2.d.06248	W03D2.7		1.24	0.57	0.06344
cea2.p.145973	F48F7.2	syn-2	1.24	0.24	0.01211
cea2.p.25822	ZC123.1		1.24	0.66	0.08277
cea2.d.13701	C18H7.2a		1.24	0.36	0.02770
cea2.d.18250	D2030.10b		1.24	0.50	0.04985
cea2.d.07439	ZC190.4		1.24	0.09	0.00160
cea2.c.34372	C37C3.13	ttr-33	1.23	0.34	0.02443
cea2.p.19216	W04A8.4		1.23	0.24	0.01266
cea2.p.106242	ZK792.4		1.22	0.24	0.01265
cea2.c.21904	M88.1	ugt-62	1.22	0.68	0.09056
cea2.p.80012	Y71H2B.7	gpa-17	1.22	0.57	0.06584
cea2.3.23100	T07H6.3	col-166	1.22	0.67	0.08693
cea2.i.03372	H25P06.4		1.22	0.58	0.06840
cea2.p.156588	Y102A11A.2		1.22	0.20	0.00908
cea2.p.140325	F13D2.1		1.21	0.75	0.10758
cea2.i.52750	F16F9.4		1.21	0.31	0.02149
cea2.d.36665	T03F7.7b		1.21	0.53	0.05890
cea2.c.42163	C43C3.1	ifp-1	1.20	0.08	0.00130
cea2.i.50105	C07A4.3		1.20	0.38	0.03185
cea2.p.122425	R10E8.8		1.20	0.38	0.03209
cea2.i.35868	F07C3.9		1.20	0.03	0.00016
cea2.d.30170	F58B3.1	lys-4	1.20	0.22	0.01099
cea2.c.34009	C16D9.1		1.20	0.70	0.09673
cea2.d.05917	T23F2.2a		1.20	0.56	0.06663
cea2.d.41881	Y105C5A.12		1.20	0.24	0.01265
cea2.p.140361	F13D11.4		1.19	0.48	0.05001
cea2.i.52115	D1053.3		1.19	0.23	0.01273
cea2.p.148226	F59F3.4		1.19	0.70	0.10025
cea2.p.103972	Y69A2AR.19		1.18	0.54	0.06222
cea2.p.90444	F13B12.3		1.18	0.71	0.10126
cea2.p.94275	F56D5.6		1.18	0.31	0.02168
cea2.p.107602	C03A7.11	ugt-51	1.17	0.50	0.05495
cea2.p.07292	F17B5.1		1.17	0.43	0.04270
cea2.p.155603	T28B4.4		1.17	0.57	0.07022
cea2.p.86439	C17H12.4		1.16	0.40	0.03724
cea2.i.00129	B0511.5	cutl-14	1.16	0.46	0.04893
cea2.c.36302	F43H9.1	ech-3	1.16	0.19	0.00895
cea2.c.39513	Y38A10A.5	crt-1	1.16	0.22	0.01129
cea2.p.90272	F11E6.8		1.15	0.40	0.03734
cea2.d.46971	Y73C8B.1		1.15	0.04	0.00039
cea2.i.23188	C01G5.9		1.15	0.40	0.03877
cea2.d.46962	Y73C8B.1		1.15	0.21	0.01062
cea2.d.19500	F10C1.8a		1.14	0.31	0.02408
cea2.i.00937	C47B2.8	prx-11	1.14	0.34	0.02766
cea2.p.71492	R74.2		1.14	0.37	0.03242

cea2.p.41539	F59B10.4		1.14	0.41	0.04095
cea2.c.10393	C32D5.9	lgg-1	1.14	0.02	0.00006
cea2.d.43211	Y35H6.1		1.14	0.23	0.01391
cea2.3.29371	Y54F10AM.2b		1.14	0.23	0.01333
cea2.c.36483	F53C11.3		1.14	0.12	0.00371
cea2.i.34120	C33G8.8	nhr-139	1.13	0.56	0.07350
cea2.p.139192	F08B12.2	prx-12	1.12	0.27	0.01851
cea2.d.44640	Y49E10.23b		1.12	0.60	0.08301
cea2.i.41012	K04A8.10		1.12	0.31	0.02432
cea2.c.13869	T01D1.3		1.12	0.22	0.01311
cea2.i.25588	F25H8.6	zbed-6	1.12	0.21	0.01141
cea2.p.10905	F47G4.3	gpdh-1	1.11	0.65	0.09800
cea2.p.142696	F29G6.1		1.11	0.28	0.02064
cea2.p.109700	C25F9.5		1.11	0.41	0.04348
cea2.i.37910	F28G4.4		1.10	0.16	0.00737
cea2.p.26034	ZC328.3		1.10	0.19	0.00974
cea2.3.26493	W06F12.2a		1.10	0.14	0.00551
cea2.p.139184	F08B12.1		1.10	0.12	0.00380
cea2.i.04852	T23H4.3	nas-5	1.10	0.03	0.00024
cea2.c.22567	T03F6.4		1.10	0.07	0.00126
cea2.p.108173	C08B6.1	ugt-17	1.10	0.26	0.01766
cea2.c.41192	C09F12.1	clc-1	1.10	0.13	0.00502
cea2.i.37138	F20G2.5		1.09	0.37	0.03686
cea2.p.145661	F48C5.1		1.09	0.43	0.04902
cea2.c.32012	Y54G2A.11		1.09	0.33	0.02862
cea2.d.40746	W01B11.4a		1.09	0.25	0.01750
cea2.d.39537	T21C12.1f		1.09	0.45	0.05188
cea2.c.45208	F47B10.7	acbp-3	1.09	0.25	0.01747
cea2.i.16017	T28D9.3		1.09	0.17	0.00845
cea2.p.40292	F52H3.5		1.09	0.20	0.01152
cea2.p.130199	Y97E10AR.2		1.08	0.37	0.03745
cea2.i.26632	F56H11.2		1.08	0.34	0.03150
cea2.d.11175	C06H5.2	fbxa-157	1.07	0.14	0.00579
cea2.c.18773	F22B7.9		1.07	0.13	0.00511
cea2.p.11033	F48C1.1a		1.06	0.47	0.05891
cea2.p.98467	T12G3.1		1.06	0.44	0.05344
cea2.3.13631	F38B7.1a		1.06	0.41	0.04711
cea2.i.50580	C15H9.10		1.06	0.24	0.01731
cea2.p.105142	Y116A8C.4		1.06	0.48	0.06114
cea2.i.21008	T24C4.4		1.06	0.16	0.00735
cea2.p.158048	ZK1193.5	dve-1	1.05	0.30	0.02532
cea2.p.131076	ZC443.3		1.05	0.27	0.02099
cea2.p.152538	T02C5.3	igcm-3	1.05	0.39	0.04247
cea2.i.23393	C08F11.7		1.05	0.30	0.02548
cea2.c.21767	K11H3.1a		1.04	0.20	0.01160
cea2.3.10303	F16B4.12b		1.04	0.27	0.02180
cea2.i.11795	F31D5.5		1.03	0.13	0.00553
cea2.p.75395	W05B2.7		1.03	0.27	0.02232
cea2.c.32570	Y73B6BR.1	pqn-89	1.03	0.13	0.00501
cea2.d.04685	M01A8.1		1.03	0.33	0.03199
cea2.c.24842	Y82E9BR.3		1.03	0.15	0.00727
cea2.c.23702	Y37D8A.16		1.03	0.06	0.00114
cea2.p.128021	Y39B6A.24		1.02	0.33	0.03272
cea2.i.04872	T27A3.5		1.02	0.39	0.04546
cea2.d.02230	F13B10.1b		1.02	0.29	0.02525
cea2.p.34765	E04F6.6		1.02	0.33	0.03385
cea2.d.46464	Y69H2.3c		1.02	0.24	0.01846
cea2.d.32024	K03H1.10		1.01	0.13	0.00531
cea2.p.155479	T27A10.7	cgr-1	1.01	0.08	0.00215

cea2.d.42404	Y119D3B.19	fbxa-78	1.01	0.34	0.03630
cea2.p.157042	ZC373.7	col-176	1.01	0.24	0.01804
cea2.i.09372	C18H9.6		1.01	0.31	0.02927
cea2.i.22855	ZK353.2		1.01	0.38	0.04358
cea2.p.123405	T04C12.4	act-3	1.01	0.13	0.00549
cea2.i.22067	Y54F10BM.12		1.00	0.24	0.01876
cea2.d.42405	Y119D3B.19	fbxa-78	1.00	0.42	0.05486
cea2.c.18694	F10E9.12		1.00	0.24	0.01892
cea2.d.21445	F20C5.2b		1.00	0.24	0.01872
cea2.p.102494	Y55F3AM.10		1.00	0.21	0.01418
cea2.i.00182	C01H6.1	col-61	0.99	0.37	0.04397
cea2.p.07633	F22G12.3		0.98	0.27	0.02410
cea2.i.59706	Y46D2A.2		0.98	0.29	0.02883
cea2.i.23814	C28C12.4		0.98	0.06	0.00138
cea2.p.110541	C37C3.6b		0.98	0.17	0.00993
cea2.p.142091	F22E10.3	pgp-14	0.97	0.51	0.08008
cea2.d.41254	W06F12.2c		0.97	0.18	0.01088
cea2.i.35212	C54D10.9		0.97	0.40	0.05217
cea2.p.121921	R02D5.3		0.96	0.07	0.00195
cea2.i.10487	E04D5.4		0.96	0.48	0.07381
cea2.c.44883	F41E7.8	cnc-8	0.96	0.26	0.02278
cea2.p.153566	T09B9.2		0.96	0.29	0.02922
cea2.p.23179	Y65B4A.2		0.96	0.21	0.01555
cea2.d.38717	T13A10.11a		0.96	0.47	0.07085
cea2.p.150992	R03A10.5		0.95	0.32	0.03603
cea2.p.143080	F31F6.5	daf-6	0.95	0.34	0.04023
cea2.p.66240	F56D2.3		0.95	0.16	0.00941
cea2.i.00219	C04F1.1		0.95	0.44	0.06461
cea2.p.142197	F23A7.6		0.95	0.42	0.06087
cea2.i.47220	Y46H3A.4		0.95	0.23	0.01855
cea2.p.113263	F16B4.2		0.95	0.43	0.06172
cea2.p.37650	F26G1.6		0.94	0.33	0.03832
cea2.3.15285	F49E10.4a		0.94	0.23	0.01902
cea2.p.03143	C32E12.4		0.94	0.20	0.01441
cea2.i.38013	F31E9.1	fbxa-180	0.94	0.46	0.07152
cea2.i.11935	F35C11.4		0.94	0.18	0.01205
cea2.d.04204	F59C6.11		0.94	0.35	0.04321
cea2.c.39363	W06H8.5		0.94	0.06	0.00153
cea2.d.21291	F18E9.6		0.93	0.44	0.06709
cea2.p.122476	R11D1.11	dhs-21	0.93	0.22	0.01823
cea2.i.29862	Y39C12A.1		0.93	0.14	0.00788
cea2.3.31044	ZK1055.6a		0.93	0.12	0.00524
cea2.i.40335	F58H1.4		0.93	0.10	0.00372
cea2.d.10726	C05D10.4c		0.93	0.07	0.00200
cea2.i.24759	C50F7.1		0.93	0.35	0.04369
cea2.c.26521	C28C12.5	spp-8	0.93	0.20	0.01582
cea2.p.57677	C18D11.2	maa-1	0.93	0.11	0.00435
cea2.p.04955	C46H11.2		0.92	0.39	0.05580
cea2.d.32028	K03H1.10		0.92	0.25	0.02317
cea2.p.123213	T03D3.5		0.92	0.20	0.01527
cea2.c.05228	M05B5.2		0.92	0.22	0.01902
cea2.p.126441	W02F12.2		0.92	0.20	0.01556
cea2.p.82349	ZK512.3	ced-11	0.92	0.22	0.01926
cea2.i.59268	F14D7.7		0.92	0.23	0.02010
cea2.p.117319	F46E10.11		0.92	0.15	0.00844
cea2.p.94515	F58G6.4	acc-1	0.91	0.11	0.00488
cea2.p.33859	C56E6.6		0.91	0.36	0.04766
cea2.i.39288	F47B8.2		0.90	0.16	0.01026
cea2.p.43984	R03D7.6	gst-5	0.90	0.23	0.02111

cea2.d.41239	W06F12.2b		0.90	0.09	0.00327
cea2.3.28746	Y48C3A.5a		0.90	0.29	0.03401
cea2.p.128388	Y43F8A.3		0.90	0.05	0.00092
cea2.d.24764	F40F12.7		0.90	0.34	0.04446
cea2.p.157330	ZK54.2	tps-1	0.90	0.21	0.01717
cea2.p.110142	C31A11.5	oac-6	0.89	0.16	0.01028
cea2.d.03397	F42G2.4a		0.89	0.19	0.01416
cea2.p.11314	F53B6.1	tsp-15	0.89	0.14	0.00857
cea2.p.12545	F56G4.1	oac-34	0.89	0.28	0.03142
cea2.p.89320	C55F2.2	ilys-4	0.89	0.29	0.03312
cea2.p.140340	F13D11.1		0.88	0.18	0.01304
cea2.p.150939	R02E12.6	hrg-1	0.88	0.28	0.03178
cea2.i.40516	F59E11.11	nhr-143	0.87	0.20	0.01629
cea2.c.41871	C30G4.7		0.87	0.17	0.01309
cea2.i.51322	C34F6.6		0.87	0.13	0.00764
cea2.p.156645	Y102A11A.3		0.87	0.29	0.03466
cea2.p.126495	W02G9.2	kel-8	0.87	0.29	0.03433
cea2.i.01271	C55C2.4		0.86	0.45	0.07984
cea2.p.51558	Y53F4B.12		0.86	0.02	0.00012
cea2.3.20115	K11H3.1b		0.86	0.16	0.01139
cea2.i.01151	C53D5.2		0.86	0.30	0.03903
cea2.i.13877	K07E1.1		0.86	0.24	0.02626
cea2.p.47606	T24B8.4		0.85	0.20	0.01769
cea2.i.53107	F22A3.5	ceh-60	0.85	0.32	0.04430
cea2.3.27003	Y105E8B.1c		0.85	0.02	0.00013
cea2.c.32313	Y67D8B.4		0.85	0.27	0.03156
cea2.p.123781	T06C12.10	cgt-1	0.85	0.13	0.00735
cea2.p.105314	ZC168.1	ncx-3	0.84	0.16	0.01150
cea2.p.148844	K02A6.3		0.84	0.13	0.00784
cea2.p.78949	Y55B1AL.1		0.83	0.08	0.00297
cea2.p.48667	W06B4.2		0.83	0.11	0.00614
cea2.p.114053	F21A3.2		0.82	0.28	0.03562
cea2.p.151397	R04E5.10	ifd-1	0.82	0.22	0.02367
cea2.c.27651	F18F11.1		0.82	0.19	0.01733
cea2.p.89386	D1046.5		0.82	0.05	0.00122
cea2.p.103068	Y62E10A.11b		0.82	0.18	0.01511
cea2.p.102720	Y57G11A.3		0.82	0.21	0.02111
cea2.d.03455	F44C8.1	cyp-33C4	0.81	0.21	0.02079
cea2.i.51127	C29F7.1		0.81	0.26	0.03321
cea2.c.36758	F58E6.1b		0.81	0.07	0.00246
cea2.i.13735	K05F1.10		0.80	0.13	0.00800
cea2.p.103078	Y62E10A.13a		0.80	0.24	0.02883
cea2.c.01753	D1037.3	ftn-2	0.80	0.12	0.00721
cea2.d.26119	F44G3.6	skr-3	0.80	0.10	0.00474
cea2.c.12601	F54C9.3		0.78	0.22	0.02563
cea2.p.117023	F44E7.10		0.78	0.15	0.01125
cea2.c.28749	F42C5.8	rps-8	0.78	0.13	0.00950
cea2.p.153748	T10B10.9		0.78	0.26	0.03426
cea2.p.146397	F52B10.1	nmy-1	0.78	0.16	0.01386
cea2.c.08892	B0334.1	ttr-18	0.78	0.18	0.01696
cea2.i.37953	F28H7.8		0.77	0.18	0.01845
cea2.i.11367	F18A12.1		0.77	0.27	0.03833
cea2.p.90417	F13B6.3		0.77	0.11	0.00706
cea2.c.27633	F13H10.6		0.77	0.08	0.00400
cea2.p.87942	C43F9.8	efn-2	0.77	0.09	0.00504
cea2.p.22922	Y54E10A.16		0.77	0.10	0.00532
cea2.p.67688	K02F3.6	ceeh-1	0.77	0.10	0.00531
cea2.c.12208	F41C3.8		0.77	0.15	0.01244
cea2.c.37767	R11G11.2	nhr-58	0.77	0.01	0.00009

cea2.d.40651	T28C6.6	col-3	0.77	0.16	0.01351
cea2.d.32620	K07B1.6a		0.77	0.22	0.02632
cea2.p.42472	K06A1.4	nhr-22	0.77	0.17	0.01527
cea2.p.110139	C29F3.7		0.76	0.12	0.00851
cea2.p.158146	B0564.6		0.76	0.06	0.00201
cea2.c.111129	F10C1.7a		0.76	0.13	0.00902
cea2.i.23103	B0496.8	tag-224	0.76	0.06	0.00191
cea2.p.139083	F02E8.6	ncr-1	0.75	0.18	0.01806
cea2.i.32821	C08B6.3		0.75	0.12	0.00811
cea2.c.48067	T20B5.2		0.74	0.16	0.01470
cea2.p.109234	C18C4.5a		0.74	0.07	0.00304
cea2.i.03334	F59C6.3		0.73	0.17	0.01718
cea2.i.17888	ZK250.5	math-47	0.73	0.09	0.00522
cea2.d.30305	F58H7.8	fbxc-3	0.72	0.06	0.00239
cea2.p.20716	Y39G10AR.10		0.72	0.15	0.01451
cea2.d.07879	F25H8.5b		0.72	0.17	0.01758
cea2.d.43111	Y32F6A.4		0.72	0.02	0.00022
cea2.d.03944	F54A5.3c		0.71	0.09	0.00517
cea2.p.11571	F54A5.2	cutl-20	0.69	0.09	0.00511
cea2.c.01661	C55B7.2	gly-2	0.64	0.05	0.00174
cea2.p.148684	H20J18.1b		0.64	0.04	0.00155
cea2.d.33455	K11D12.10b		0.62	0.01	0.00003

Table 3A-2

Genes downregulated in *acs-3(ft5)* mutant

C. elegans (07/14/09)	Gene Ids	Gene Name	Average log2 change	Std Dev	P value
cea2.p.124717	T13F3.6		-4.54	1.02	0.01635
cea2.p.50848	Y48E1B.8		-4.04	1.51	0.04354
cea2.p.95102	K02D7.3	col-101	-4.00	0.92	0.01698
cea2.p.113212	F15E11.12		-3.81	0.72	0.01178
cea2.i.45823	W06G6.11		-3.73	1.16	0.03068
cea2.3.24008	T15D6.3	gly-17	-3.73	0.82	0.01584
cea2.p.158386	F15E11.14		-3.55	0.96	0.02341
cea2.p.132864	C05E11.4	amt-1	-3.48	0.74	0.01457
cea2.i.18516	C02D5.3	gsto-2	-3.48	0.16	0.00071
cea2.p.124718	T15B7.3	col-143	-3.11	1.46	0.06635
cea2.i.42675	R09B5.4	fpn-1.2	-3.07	0.66	0.01518
cea2.p.87235	C32H11.12	dod-24	-2.92	0.58	0.01301
cea2.d.30327	F59D8.1	vit-3	-2.88	0.42	0.00714
cea2.p.11540	F53G12.7	col-45	-2.88	0.56	0.01229
cea2.p.111863	D1086.3		-2.84	1.28	0.06116
cea2.p.154713	T22B7.7		-2.76	0.47	0.00960
cea2.i.39348	F47C10.6	ugt-32	-2.68	0.73	0.02373
cea2.i.53381	F31B9.2		-2.62	1.50	0.09458
cea2.d.20845	F15E11.1		-2.59	0.42	0.00850
cea2.p.154841	T22H6.7	abf-6	-2.58	1.12	0.05757
cea2.i.54251	F49H12.2		-2.57	0.83	0.03326
cea2.p.111924	D2023.7	col-158	-2.55	0.81	0.03176
cea2.p.78886	Y54F10AR.2		-2.55	0.34	0.00584
cea2.i.57977	Y40A1A.1		-2.53	1.58	0.10960
cea2.d.26120	F45D11.14		-2.52	0.65	0.02145
cea2.i.00585	C27C7.3	nhr-74	-2.41	0.80	0.03504
cea2.d.06866	Y50D4B.4		-2.40	0.48	0.01297
cea2.i.16393	W10G11.3		-2.39	0.87	0.04138
cea2.i.15550	T16A1.4		-2.37	0.96	0.05084
cea2.i.47415	Y49G5A.1		-2.35	0.98	0.05310
cea2.d.39373	T20D4.4		-2.34	1.03	0.05952
cea2.d.00307	C05D10.1b		-2.33	0.40	0.00950
cea2.p.153685	T10B10.3		-2.28	0.04	0.00011
cea2.d.11950	C09G12.5		-2.25	0.60	0.02316
cea2.i.39543	F49A5.7	clec-33	-2.25	1.11	0.07234
cea2.i.10379	D1022.3		-2.24	0.21	0.00293
cea2.c.17748	C30D11.1	unc-103	-2.24	0.64	0.02651
cea2.3.28999	Y50D4B.4		-2.23	0.10	0.00070
cea2.p.158050	C04E12.4		-2.22	0.63	0.02554
cea2.i.56133	R01E6.2		-2.19	1.45	0.12071
cea2.p.110781	C43D7.2	fbxb-65	-2.15	1.30	0.10357
cea2.3.05195	C35A5.3		-2.15	0.13	0.00123
cea2.d.31787	K02A2.1		-2.13	0.46	0.01514
cea2.i.24189	C39E9.9	col-132	-2.06	1.73	0.17566
cea2.p.50376	Y48B6A.4	eat-2	-2.05	1.01	0.07295
cea2.d.11947	C09G12.5		-2.04	0.54	0.02242
cea2.c.49390	C52D10.6	skr-12	-2.02	0.66	0.03390
cea2.d.07957	F57C9.1a		-2.02	0.80	0.04872
cea2.p.75766	Y22D7AR.10		-2.02	1.19	0.09860
cea2.d.07549	ZK512.7		-2.00	0.10	0.00087
cea2.p.29997	C09G5.3	col-79	-2.00	2.01	0.22710

cea2.p.89211	C53B4.5	col-119	-2.00	0.73	0.04191
cea2.i.56646	T01B10.1	grd-4	-1.98	0.95	0.06869
cea2.p.66324	F56D2.8		-1.98	0.87	0.05873
cea2.d.44122	Y45G12C.12	str-154	-1.98	0.51	0.02118
cea2.d.04672	K12H6.9		-1.97	0.72	0.04154
cea2.p.42029	K01A2.3a		-1.96	0.52	0.02235
cea2.p.31798	C30G12.2		-1.94	1.48	0.15023
cea2.d.38506	T10B5.8		-1.92	0.99	0.07896
cea2.i.23982	C32H11.3		-1.91	1.02	0.08278
cea2.i.30131	Y43B11AL.3	srw-64	-1.91	0.67	0.03884
cea2.d.07339	Y73B6BL.24		-1.91	0.69	0.04067
cea2.i.27268	K03H6.4		-1.90	0.71	0.04309
cea2.i.51256	C33D3.3		-1.89	0.89	0.06587
cea2.d.07747	C07A9.6b		-1.89	0.29	0.00775
cea2.d.30360	F59D8.1	vit-3	-1.89	0.69	0.04187
cea2.p.149938	K09F5.2	vit-1	-1.88	0.85	0.06112
cea2.i.27375	K08D12.6		-1.88	0.69	0.04230
cea2.i.59232	C52E2.8		-1.85	1.05	0.09187
cea2.d.46340	Y69A2AR.12		-1.84	0.63	0.03666
cea2.i.58494	ZK1086.2		-1.83	1.04	0.09318
cea2.d.08105	Y47G6A.20c		-1.81	1.31	0.13811
cea2.i.31044	Y69A2AR.25		-1.80	0.47	0.02151
cea2.d.39379	T20D4.7		-1.80	0.50	0.02522
cea2.d.07897	F35D11.2b		-1.80	0.66	0.04261
cea2.i.55662	K09A11.2	cyp-14A1	-1.79	0.78	0.05726
cea2.d.08095	Y37D8A.23a		-1.78	0.62	0.03846
cea2.p.159335	F22A3.6	ilys-5	-1.76	0.77	0.05744
cea2.3.03224	C17B7.5		-1.76	0.98	0.09049
cea2.i.38631	F38E1.6		-1.75	0.75	0.05659
cea2.d.00892	C24G6.2b		-1.75	0.76	0.05717
cea2.i.44271	T09H2.1	cyp-34A4	-1.73	0.42	0.01904
cea2.i.59038	Y50D4B.3		-1.72	0.46	0.02305
cea2.c.37003	K02E2.4	ins-35	-1.71	0.42	0.01919
cea2.d.33301	K10D6.2c		-1.70	0.98	0.09524
cea2.d.07838	F01F1.10a		-1.70	1.10	0.11633
cea2.p.81047	Y111B2A.14	pqn-80	-1.69	1.07	0.11229
cea2.i.53228	F23D12.1		-1.69	0.35	0.01422
cea2.3.29007	Y50D4B.4		-1.68	0.28	0.00891
cea2.c.41919	C34F6.2	col-178	-1.67	0.19	0.00439
cea2.d.46355	Y69A2AR.13		-1.66	0.13	0.00198
cea2.d.05181	T01D1.2a		-1.65	0.80	0.07046
cea2.p.36165	F11G11.11	col-20	-1.64	0.32	0.01251
cea2.i.31002	Y67D8C.7		-1.63	0.96	0.09917
cea2.3.08988	F11A1.3a		-1.62	0.88	0.08506
cea2.d.31982	K03D3.5		-1.62	0.81	0.07375
cea2.d.10485	C04E12.5		-1.61	0.55	0.03659
cea2.p.136035	C34F6.3	col-179	-1.61	0.18	0.00397
cea2.i.43403	T03D3.1	ugt-53	-1.61	0.39	0.01876
cea2.d.44373	Y47H9A.1		-1.60	0.65	0.05026
cea2.i.47322	Y47D7A.12		-1.60	0.69	0.05725
cea2.i.27370	K08D12.6		-1.60	0.56	0.03902
cea2.d.10465	C04E12.11		-1.60	0.36	0.01612
cea2.i.59271	F15E6.8	dct-7	-1.60	0.62	0.04604
cea2.d.31850	K02E7.9	btb-10	-1.59	1.29	0.16704
cea2.i.40964	K03B4.6		-1.59	0.45	0.02618
cea2.i.18681	C09F5.3		-1.58	0.69	0.05779
cea2.i.52776	F16H9.2	nhr-27	-1.56	1.13	0.13956
cea2.c.47712	T10B10.2	ucr-2.2	-1.56	0.28	0.01091
cea2.d.33155	K09H9.1		-1.55	0.96	0.10652

cea2.i.28889	W08E12.6		-1.55	0.87	0.09179
cea2.d.16110	C39D10.8b		-1.54	0.42	0.02368
cea2.c.42461	C49F5.7		-1.54	0.93	0.10281
cea2.d.24444	F39B2.4a		-1.53	0.29	0.01194
cea2.i.38628	F38E1.11		-1.53	0.15	0.00332
cea2.p.139499	F09E10.1		-1.53	0.85	0.08957
cea2.c.44218	F21E9.4	ins-39	-1.53	1.23	0.16277
cea2.c.49373	ZK1193.1	col-19	-1.52	0.19	0.00495
cea2.d.21817	F23H11.8a		-1.52	0.35	0.01771
cea2.c.33961	C15C8.2	cky-1	-1.50	0.55	0.04279
cea2.p.81273	ZC21.3		-1.49	1.00	0.12230
cea2.d.24495	F39B2.4b		-1.48	0.18	0.00496
cea2.d.05214	T02G5.9b		-1.48	0.49	0.03524
cea2.i.48639	ZC412.1		-1.48	0.59	0.04935
cea2.p.102136	Y54G2A.6	clec-85	-1.48	1.18	0.16163
cea2.i.24912	C54E4.5		-1.46	0.26	0.01043
cea2.d.02156	F10C1.2a		-1.46	0.46	0.03189
cea2.d.39815	T23D8.9b		-1.45	0.09	0.00136
cea2.i.10818	F09E5.13	agt-2	-1.45	0.24	0.00877
cea2.d.39813	T23D8.9b		-1.44	0.36	0.01990
cea2.i.27436	K10D11.2		-1.43	0.38	0.02318
cea2.p.124499	T10C6.7		-1.42	0.25	0.01043
cea2.i.16454	Y110A2AM.1		-1.42	0.80	0.09198
cea2.d.21714	F22E5.5	sri-52	-1.41	0.85	0.10385
cea2.d.32222	K04G2.8a		-1.41	0.33	0.01825
cea2.c.35201	F10D2.9	fat-7	-1.41	0.59	0.05353
cea2.p.49633	Y38E10A.22		-1.40	0.43	0.03010
cea2.p.150193	K10C2.3		-1.40	0.43	0.03064
cea2.d.16119	C39D10.8c		-1.40	0.47	0.03629
cea2.p.60369	C56G2.15		-1.40	0.34	0.01900
cea2.d.17624	C52D10.8	skr-13	-1.39	0.78	0.09128
cea2.p.69922	R02F2.8		-1.39	0.31	0.01578
cea2.p.115085	F26F12.1	col-140	-1.39	0.15	0.00411
cea2.c.49420	Y111B2A.2		-1.38	0.23	0.00883
cea2.p.124403	T09F5.9	clec-47	-1.37	0.24	0.01016
cea2.i.36382	F13A2.3		-1.37	0.50	0.04207
cea2.i.42786	R11D1.3		-1.36	0.04	0.00025
cea2.c.07484	Y47H9C.4a		-1.36	0.62	0.06378
cea2.p.30002	C09G5.5	col-80	-1.35	0.26	0.01216
cea2.d.07882	F29C4.7a		-1.34	0.30	0.01672
cea2.p.158092	F43G6.7		-1.34	0.27	0.01321
cea2.c.40280	Y113G7B.23	psa-1	-1.33	0.71	0.08302
cea2.c.49425	Y60A3A.12	chk-2	-1.33	0.81	0.10450
cea2.d.36725	T04A11.3		-1.32	0.24	0.01094
cea2.3.06804	C49H3.5a		-1.32	0.63	0.06780
cea2.i.43687	T05E12.2	srbc-14	-1.32	0.87	0.12105
cea2.3.06753	C49D10.1		-1.31	0.23	0.01050
cea2.d.42799	Y19D10A.9	clec-209	-1.30	0.40	0.03062
cea2.p.154095	T13H2.5	spat-3	-1.29	0.12	0.00284
cea2.i.24881	C54E4.1		-1.29	0.62	0.06907
cea2.i.35583	D1065.5	srh-211	-1.28	0.68	0.08231
cea2.p.106915	B0213.14	cyp-34A8	-1.28	0.35	0.02418
cea2.p.134935	C25F6.1		-1.28	0.26	0.01398
cea2.d.06710	Y46H3B.2	clec-204	-1.27	0.38	0.02858
cea2.i.17738	ZK1067.8		-1.27	0.16	0.00526
cea2.p.148596	H13N06.6	tbh-1	-1.27	0.65	0.07778
cea2.i.61252	F01D5.2		-1.27	0.26	0.01406
cea2.i.43964	T07C12.8		-1.27	0.47	0.04334
cea2.i.58846	F11D5.4		-1.26	0.43	0.03739

cea2.i.53072	F21E9.6		-1.26	0.99	0.15748
cea2.i.12394	F43C11.3		-1.26	0.53	0.05380
cea2.i.16474	Y17G7B.1	acbp-6	-1.26	0.13	0.00335
cea2.i.25158	F08G5.5	ugt-65	-1.25	0.22	0.01036
cea2.c.32710	Y116A8C.32	sfa-1	-1.25	0.22	0.01002
cea2.c.43137	F09C8.1		-1.25	0.43	0.03786
cea2.p.43957	R03D7.2		-1.25	0.17	0.00628
cea2.c.41121	C08A9.1	sod-3	-1.25	0.51	0.05219
cea2.3.19022	K06B9.4		-1.24	0.45	0.04049
cea2.i.14539	R07C3.10	fbxc-27	-1.23	0.45	0.04184
cea2.i.58729	ZK899.1		-1.23	0.39	0.03128
cea2.p.157812	ZK813.1		-1.23	0.33	0.02290
cea2.d.34165	M04C9.6b		-1.23	0.38	0.02983
cea2.d.37493	T06E4.3a		-1.23	0.59	0.06906
cea2.i.08550	C03H5.1	clcc-10	-1.23	0.28	0.01743
cea2.p.20305	Y23H5B.7b		-1.22	0.27	0.01615
cea2.i.21713	Y39A3B.3		-1.22	0.84	0.12788
cea2.c.18330	F01F1.6	alh-9	-1.22	0.39	0.03314
cea2.i.48616	ZC404.7		-1.22	0.03	0.00025
cea2.i.19179	D1044.5		-1.21	0.26	0.01484
cea2.p.87222	C32H11.4		-1.21	0.14	0.00419
cea2.c.50260	C49G7.4	phat-3	-1.21	0.07	0.00112
cea2.p.86637	C24F3.6	col-124	-1.21	0.43	0.03949
cea2.i.58702	ZK813.2		-1.20	0.52	0.05755
cea2.d.31866	K02F2.1b		-1.20	0.45	0.04453
cea2.p.150430	M02F4.8	aqp-7	-1.19	0.36	0.02922
cea2.p.146461	F52D2.4	gei-12	-1.19	0.18	0.00748
cea2.d.16401	C42C1.4		-1.19	0.41	0.03682
cea2.3.13761	F40G12.11		-1.18	0.43	0.04161
cea2.c.18698	F11H8.3	col-8	-1.18	0.81	0.12824
cea2.d.01668	C48G7.1		-1.18	0.30	0.02036
cea2.i.36175	F10C2.7		-1.18	0.19	0.00820
cea2.p.69392	M88.4		-1.18	0.15	0.00511
cea2.c.43172	F09E10.6		-1.17	0.12	0.00350
cea2.3.16245	F54E7.3b		-1.17	0.11	0.00274
cea2.i.30617	Y55F3AR.3	cct-8	-1.16	0.08	0.00164
cea2.p.34911	F01D5.7		-1.16	0.47	0.05031
cea2.d.07777	C24H12.5a		-1.16	0.18	0.00755
cea2.d.05735	T19A5.2a		-1.16	0.24	0.01419
cea2.i.32970	C10G8.7	ceh-33	-1.15	0.11	0.00295
cea2.c.36418	F46E10.2		-1.15	0.32	0.02520
cea2.i.33047	C12D5.9		-1.15	0.08	0.00175
cea2.d.07946	F54B11.3b		-1.15	0.74	0.11473
cea2.d.01913	E02H4.3b		-1.15	0.66	0.09401
cea2.i.47538	Y50E8A.14		-1.15	0.48	0.05291
cea2.p.59134	C36A4.2	cyp-25A2	-1.15	0.51	0.06019
cea2.i.17739	ZK1127.7	cin-4	-1.15	0.19	0.00867
cea2.p.69261	M03C11.3		-1.15	0.27	0.01801
cea2.p.66660	F58A4.1		-1.15	0.47	0.05234
cea2.c.50416	C33H5.4	klp-10	-1.15	0.56	0.07142
cea2.i.61248	F01D5.2		-1.14	0.07	0.00134
cea2.d.07741	C06A8.1b		-1.14	0.49	0.05569
cea2.i.61249	F01D5.2		-1.13	0.10	0.00277
cea2.i.14251	M151.3		-1.13	0.66	0.09720
cea2.d.00791	C23H3.2a		-1.13	0.19	0.00926
cea2.i.17599	Y59C2A.2		-1.13	0.18	0.00882
cea2.p.102537	Y55F3AR.2		-1.12	0.13	0.00467
cea2.i.24080	C33H5.6		-1.12	0.45	0.04884
cea2.i.30638	Y55F3BL.2		-1.12	0.45	0.04992

cea2.p.129713	Y60A3A.10	dhs-24	-1.12	0.54	0.06978
cea2.3.31169	ZK1127.7	cin-4	-1.11	0.24	0.01510
cea2.i.27429	K10D11.1	dod-17	-1.11	0.37	0.03552
cea2.c.16150	B0244.2	ida-1	-1.11	0.05	0.00061
cea2.d.47718	ZC404.3b		-1.11	0.28	0.02116
cea2.i.39618	F52F10.4	oac-32	-1.11	0.40	0.04013
cea2.3.04056	C25G4.10		-1.11	0.41	0.04251
cea2.i.40843	K02E11.6		-1.10	0.44	0.04858
cea2.d.07190	Y67D8A.2a		-1.10	0.49	0.05970
cea2.i.57929	Y12A6A.1		-1.10	0.48	0.05775
cea2.i.59619	T28A11.2		-1.10	0.37	0.03545
cea2.i.23798	C27H2.2		-1.09	0.44	0.05076
cea2.i.32115	B0507.10		-1.09	0.17	0.00787
cea2.i.27460	K11H12.4		-1.08	0.38	0.03892
cea2.d.13978	C24B9.13	srx-25	-1.08	0.31	0.02693
cea2.3.05273	C36A4.1	cyp-25A1	-1.08	0.38	0.03876
cea2.d.00580	C09H6.2a		-1.08	0.42	0.04677
cea2.c.11714	F28C6.10		-1.06	0.25	0.01859
cea2.i.50964	C25A11.1		-1.06	0.26	0.01942
cea2.i.29836	Y38F2AR.5		-1.06	0.11	0.00357
cea2.d.14478	C26E6.9c		-1.06	0.31	0.02755
cea2.p.116548	F40G12.11		-1.06	0.27	0.02086
cea2.i.56514	R09F10.6	srh-11	-1.05	0.52	0.07243
cea2.p.109143	C17E7.12		-1.05	0.28	0.02309
cea2.d.03982	F55A8.2c		-1.05	0.18	0.00950
cea2.i.42347	R05D8.7		-1.05	0.28	0.02238
cea2.d.11081	C06E1.5	fip-3	-1.05	0.33	0.03136
cea2.d.19909	F11C7.3a		-1.04	0.25	0.01813
cea2.d.09507	B0410.2b		-1.04	0.44	0.05566
cea2.3.23467	T09E8.1a		-1.04	0.25	0.01795
cea2.d.11946	C09F9.4		-1.04	0.05	0.00064
cea2.p.12511	F56C11.4		-1.04	0.35	0.03495
cea2.d.12928	C16A3.1c		-1.04	0.20	0.01183
cea2.i.16382	W10G11.17		-1.04	0.46	0.05887
cea2.c.25497	ZK637.13	glb-1	-1.03	0.25	0.01898
cea2.d.10479	C04E12.12		-1.03	0.08	0.00195
cea2.d.46349	Y69A2AR.12		-1.03	0.22	0.01513
cea2.d.00230	C01G5.2	prg-2	-1.03	0.21	0.01347
cea2.p.106225	ZK792.2	inx-8	-1.02	0.10	0.00333
cea2.p.129394	Y51A2D.18		-1.02	0.09	0.00260
cea2.p.36300	F13H8.3		-1.02	0.19	0.01142
cea2.d.00912	C25H3.7a		-1.01	0.07	0.00176
cea2.d.18051	D1022.7a		-1.01	0.06	0.00117
cea2.d.08178	F35D6.1b		-1.01	0.35	0.03701
cea2.3.19887	K10G9.1		-1.01	0.16	0.00871
cea2.p.139854	F11C7.2		-1.01	0.26	0.02085
cea2.d.05410	T07D4.4a		-1.01	0.16	0.00805
cea2.d.44971	Y53C12B.3a		-1.00	0.31	0.03125
cea2.c.26219	C06G3.11	tin-9.1	-1.00	0.10	0.00344
cea2.p.79920	Y71H2B.1		-0.99	0.11	0.00413
cea2.p.78014	Y48G9A.1		-0.98	0.57	0.09615
cea2.d.31596	H42K12.1b		-0.98	0.24	0.01851
cea2.i.37918	F28H7.1	srj-22	-0.98	0.58	0.09940
cea2.3.04255	C26H9A.2		-0.97	0.31	0.03207
cea2.d.37261	T05H10.5a		-0.97	0.35	0.04136
cea2.i.46171	Y113G7B.18	mdt-17	-0.97	0.27	0.02409
cea2.p.86443	C17H12.8		-0.97	0.24	0.01997
cea2.d.08325	AC8.1	pme-6	-0.97	0.48	0.07296
cea2.d.17126	C46G7.4a		-0.97	0.31	0.03178

cea2.i.01021	C48B6.9		-0.96	0.21	0.01581
cea2.c.07595	Y52B11A.8		-0.96	0.41	0.05580
cea2.3.06096	C44E4.1a		-0.96	0.38	0.04903
cea2.i.58982	T17A3.3	fbxb-81	-0.95	0.36	0.04357
cea2.p.63786	F40F12.5b		-0.95	0.45	0.06794
cea2.d.14695	C29A12.3b		-0.94	0.31	0.03475
cea2.i.20175	K11H3.5		-0.94	0.30	0.03243
cea2.p.90605	F15E6.4		-0.93	0.17	0.01109
cea2.d.05318	T05C1.6		-0.93	0.23	0.02000
cea2.d.21019	F17E9.12	his-31	-0.93	0.22	0.01805
cea2.d.25467	F43G6.1b		-0.93	0.21	0.01671
cea2.i.33249	C13F10.2		-0.92	0.20	0.01500
cea2.p.103923	Y69A2AR.17		-0.92	0.26	0.02490
cea2.p.119166	F57B1.6		-0.92	0.15	0.00873
cea2.p.156940	ZC53.7	rgs-9	-0.92	0.38	0.05256
cea2.p.33109	C47D12.4		-0.91	0.18	0.01216
cea2.i.29320	Y11D7A.13	flh-3	-0.91	0.49	0.08281
cea2.d.02591	F20G4.2		-0.91	0.15	0.00877
cea2.p.48427	W02B8.2		-0.91	0.14	0.00743
cea2.d.10467	C04E12.11		-0.91	0.14	0.00743
cea2.i.30643	Y55F3BL.3		-0.91	0.28	0.03079
cea2.c.01371	C44E4.4		-0.90	0.15	0.00909
cea2.p.06753	F11C3.2	unc-122	-0.90	0.12	0.00632
cea2.i.24741	C50A2.2		-0.90	0.07	0.00190
cea2.d.41356	W06H8.1e		-0.90	0.07	0.00190
cea2.p.128120	Y39B6A.38	reps-1	-0.90	0.18	0.01340
cea2.i.31210	Y73F8A.18		-0.89	0.13	0.00742
cea2.d.30363	F59D8.1	vit-3	-0.89	0.26	0.02796
cea2.p.131528	ZK856.12		-0.89	0.15	0.00955
cea2.d.05764	T19H12.10	ugt-11	-0.89	0.15	0.00897
cea2.p.155406	T26C11.7	ceh-39	-0.88	0.19	0.01456
cea2.p.156679	Y102A11A.8		-0.88	0.15	0.00931
cea2.p.24411	Y92H12A.5		-0.87	0.29	0.03411
cea2.d.07967	H18N23.2b		-0.87	0.17	0.01260
cea2.d.03380	F42A9.1a		-0.87	0.25	0.02594
cea2.d.07303	Y71H10B.1a		-0.86	0.28	0.03319
cea2.3.06163	C44E4.7		-0.86	0.34	0.04776
cea2.d.08092	Y34D9A.3b		-0.86	0.22	0.02193
cea2.d.08024	R148.5a		-0.86	0.08	0.00310
cea2.d.07951	F55G11.3		-0.85	0.03	0.00042
cea2.d.06370	W09C2.3a		-0.85	0.14	0.00942
cea2.i.53604	F38B6.2		-0.85	0.23	0.02442
cea2.d.15087	C32A3.1a		-0.85	0.24	0.02463
cea2.d.05734	T18H9.7a		-0.85	0.12	0.00664
cea2.d.23816	F36A2.1a		-0.85	0.12	0.00635
cea2.d.37520	T06E4.3b		-0.85	0.06	0.00171
cea2.d.07950	F55B12.3a		-0.85	0.05	0.00133
cea2.d.07866	F19F10.11b		-0.84	0.16	0.01212
cea2.d.23834	F36A2.1b		-0.84	0.19	0.01755
cea2.p.88987	C50F7.4		-0.83	0.12	0.00635
cea2.i.05266	W04A8.6		-0.83	0.22	0.02248
cea2.d.25933	F44E2.2c		-0.83	0.26	0.03049
cea2.p.109123	C17E7.4		-0.82	0.19	0.01832
cea2.i.31737	ZK792.3	inx-9	-0.82	0.18	0.01558
cea2.i.50388	C11H1.5		-0.82	0.24	0.02718
cea2.i.41733	K11G9.2		-0.81	0.10	0.00514
cea2.p.51923	Y54G11A.2		-0.81	0.07	0.00231
cea2.p.64061	F42G9.7	snt-2	-0.81	0.12	0.00721
cea2.d.03643	F48E8.7a		-0.80	0.08	0.00302

cea2.d.08189	Y34F4.3		-0.80	0.28	0.03939
cea2.p.85372	C06A6.4		-0.79	0.18	0.01586
cea2.d.46837	Y73B3A.4		-0.79	0.21	0.02276
cea2.p.137713	C49F5.6		-0.78	0.12	0.00771
cea2.i.22819	ZK112.3		-0.78	0.09	0.00409
cea2.3.01993	C07E3.1b		-0.78	0.17	0.01610
cea2.d.39804	T23D8.5	his-67	-0.77	0.04	0.00080
cea2.i.65970	Y47H9C.14		-0.77	0.18	0.01808
cea2.d.16563	C43E11.6d		-0.76	0.07	0.00245
cea2.p.133137	C07B5.5	nuc-1	-0.76	0.02	0.00034
cea2.d.31659	JC8.10b		-0.76	0.15	0.01253
cea2.p.81415	ZC155.4		-0.76	0.12	0.00794
cea2.d.16268	C40H5.6		-0.74	0.13	0.01016
cea2.c.03286	F33H2.3		-0.74	0.10	0.00640
cea2.i.26526	F55G11.2		-0.73	0.06	0.00224
cea2.p.55223	B0393.3		-0.72	0.12	0.00906
cea2.d.46741	Y71H2AM.20b		-0.72	0.13	0.01048
cea2.3.14068	F43C1.2b		-0.71	0.15	0.01538
cea2.d.14193	C25A8.1		-0.66	0.03	0.00092

Table 3A-3

Genes upregulated with *nhr-25* RNAi

C. elegans (07/14/09)	Gene Ids	Gene Name	Average log2 change	Std Dev	P value
cea2.c.37695	R09B5.9	cnc-4	6.95	0.43	0.00127
cea2.d.00017	B0213.17	nlp-34	6.71	0.17	0.00023
cea2.d.04924	R09B5.3	cnc-2	6.47	0.14	0.00015
cea2.i.26654	F57H12.3	ora-1	6.33	0.36	0.00106
cea2.i.11035	F12A10.7		6.18	0.84	0.00617
cea2.i.52302	F02D10.1	col-183	6.12	0.44	0.00170
cea2.d.16966	C45G7.3	ilys-3	6.09	1.15	0.01175
cea2.i.13348	F58E1.6		5.86	1.68	0.02648
cea2.c.03774	F48C1.9		5.79	0.32	0.00100
cea2.d.03943	F53H2.2	cnc-7	5.79	0.21	0.00044
cea2.p.87872	C42D4.3		5.77	0.68	0.00459
cea2.p.11392	F53B6.8	fipr-26	5.56	1.51	0.02360
cea2.c.34115	C24B9.9	dod-3	5.54	0.77	0.00631
cea2.p.11393	F53B6.9	fip-6	5.27	0.92	0.00994
cea2.i.38201	F32G8.3		5.23	0.54	0.00359
cea2.i.32282	C02E7.1		5.20	1.52	0.02742
cea2.d.00018	B0213.5	nlp-30	5.19	0.13	0.00020
cea2.p.87524	C34D4.15	col-113	5.10	1.01	0.01272
cea2.i.53522	F35B3.4		5.01	1.23	0.01962
cea2.d.16965	C45B2.8		4.92	0.34	0.00157
cea2.i.54615	F54B11.2	col-44	4.88	0.94	0.01213
cea2.c.33210	B0213.6	nlp-31	4.85	0.62	0.00538
cea2.p.145574	F47E1.2		4.84	0.22	0.00069
cea2.c.05339	R11A5.3		4.74	0.97	0.01361
cea2.i.47134	Y43F8C.1	nlp-25	4.67	0.91	0.01231
cea2.i.02858	F48C1.8		4.60	1.07	0.01749
cea2.p.32647	C38C6.6	tag-297	4.58	1.24	0.02347
cea2.p.53061	ZK84.1		4.46	1.88	0.05421
cea2.p.88062	C45G7.2	ilys-2	4.30	0.82	0.01185
cea2.c.38593	T19B10.2		4.24	0.78	0.01107
cea2.i.21642	Y37B11A.1	cutl-23	4.21	1.14	0.02364
cea2.p.24495	Y95B8A.2		4.21	0.74	0.01026
cea2.p.117672	F52F10.2		4.18	1.21	0.02671
cea2.p.113075	F15B9.1	far-3	4.16	0.38	0.00278
cea2.d.41508	W08E12.3		4.16	1.26	0.02938
cea2.i.08538	C01G6.9		4.09	1.68	0.05169
cea2.c.10341	C30B5.6		4.07	1.92	0.06679
cea2.c.10011	C17C3.4	ins-11	4.06	1.19	0.02724
cea2.i.34678	C49G7.5		4.00	2.60	0.11676
cea2.d.24658	F39D8.1a	pqn-36	3.98	0.25	0.00129
cea2.p.107913	C05E4.14		3.96	1.52	0.04599
cea2.p.64694	F44E2.4		3.95	0.97	0.01966
cea2.p.89237	C53B4.8		3.94	0.50	0.00530
cea2.d.03076	F35C5.10	nsps-11	3.92	1.15	0.02756
cea2.p.111517	C54F6.5		3.91	0.21	0.00096
cea2.d.01404	C45B2.2		3.91	0.14	0.00040
cea2.i.58840	F09F7.8	nsps-12	3.87	0.51	0.00573
cea2.p.128218	Y39H10A.1		3.83	0.80	0.01411
cea2.3.30545	Y73F8A.8	pqn-90	3.80	0.35	0.00280
cea2.p.101026	Y38C1BA.3	col-109	3.79	1.37	0.04073
cea2.c.35321	F21H7.12		3.75	0.46	0.00494

cea2.c.45618	F53A9.8		3.71	0.15	0.00057
cea2.i.39600	F52E1.8		3.70	2.06	0.08989
cea2.i.26088	F42C5.3		3.70	0.33	0.00258
cea2.p.139449	F09B9.1	oac-14	3.70	0.43	0.00455
cea2.d.08484	B0213.3	nlp-28	3.69	1.55	0.05394
cea2.p.110332	C35A5.2	ugt-33	3.65	0.75	0.01383
cea2.p.107362	B0507.8		3.65	0.64	0.01018
cea2.c.32759	ZC168.5	grl-21	3.63	2.06	0.09230
cea2.c.25574	ZK1010.7	col-97	3.59	1.38	0.04557
cea2.i.00516	C24G7.1		3.58	1.53	0.05599
cea2.i.28886	W08E12.2		3.57	2.37	0.12111
cea2.i.42032	M03F8.1		3.54	1.63	0.06363
cea2.p.147751	F57C12.1	nas-38	3.54	1.56	0.05919
cea2.c.34876	C54F6.14	ftn-1	3.53	0.17	0.00077
cea2.d.10280	C03A7.8	abu-7	3.51	1.10	0.03134
cea2.p.37624	F26G1.5		3.49	1.00	0.02608
cea2.i.44113	T08G5.1		3.49	0.86	0.01953
cea2.i.19343	F22B7.4	fip-1	3.47	1.73	0.07363
cea2.d.10234	C03A7.14	abu-8	3.46	1.09	0.03166
cea2.c.36886	F59E11.7		3.46	0.90	0.02160
cea2.3.15352	F49H6.5		3.45	1.88	0.08672
cea2.c.44495	F35A5.3	abu-10	3.44	1.20	0.03813
cea2.p.22926	Y54E10BL.2	col-48	3.42	1.23	0.04028
cea2.p.123158	T02E9.2		3.42	0.84	0.01976
cea2.d.10232	C03A7.14	abu-8	3.41	1.32	0.04624
cea2.d.09988	C01G12.6	nspb-10	3.40	0.44	0.00549
cea2.d.10251	C03A7.4	pqn-5	3.40	0.98	0.02654
cea2.i.54486	F53A9.7		3.38	0.27	0.00217
cea2.p.111104	C49G7.10		3.38	0.74	0.01548
cea2.p.157687	ZK662.2		3.38	1.09	0.03293
cea2.i.14934	T05C1.1		3.37	0.90	0.02291
cea2.c.11224	F13D12.3		3.36	0.33	0.00311
cea2.i.33706	C24B5.3	ptr-1	3.35	2.30	0.12771
cea2.i.41492	K09D9.1		3.35	0.09	0.00025
cea2.p.41216	F56D3.1		3.34	1.29	0.04650
cea2.i.51713	C45B2.3		3.32	0.93	0.02499
cea2.3.19098	K07B1.8		3.32	1.37	0.05225
cea2.p.43886	M195.3	amt-3	3.31	1.36	0.05189
cea2.d.10265	C03A7.7	abu-6	3.30	1.24	0.04379
cea2.p.158093	F48G7.5		3.29	1.10	0.03505
cea2.d.08308	AC3.3	abu-1	3.28	1.24	0.04477
cea2.p.68358	K08E3.1	tyr-2	3.28	0.72	0.01592
cea2.c.49413	R12E2.7		3.26	1.00	0.02998
cea2.i.22348	Y71H2AM.16		3.26	1.11	0.03666
cea2.c.17582	C29E4.1	col-90	3.24	1.60	0.07271
cea2.d.24690	F39D8.1b	pqn-36	3.23	0.37	0.00443
cea2.p.121319	K11D12.4	cpt-4	3.22	0.54	0.00916
cea2.c.33506	C02E7.7		3.21	1.35	0.05387
cea2.p.132982	C06G1.1		3.21	1.29	0.04973
cea2.c.26266	C08F11.13		3.21	0.79	0.01970
cea2.i.14193	M02G9.3		3.19	0.52	0.00872
cea2.d.47041	Y73F8A.9	pqn-91	3.18	0.78	0.01929
cea2.p.88082	C46A5.3		3.18	1.25	0.04766
cea2.i.35629	D2063.1		3.15	0.55	0.00993
cea2.i.33178	C13D9.1	srr-6	3.14	1.35	0.05615
cea2.p.53984	ZK1067.7	pqn-95	3.13	1.16	0.04317
cea2.c.28931	F49F1.5		3.12	0.14	0.00072
cea2.p.47452	T21B10.6	cutl-15	3.12	1.34	0.05594
cea2.i.38512	F36H9.2		3.12	0.69	0.01576

cea2.i.23206	C02F4.4		3.12	0.44	0.00652
cea2.p.18517	T27F6.8		3.11	0.05	0.00008
cea2.c.03270	F33D11.8		3.11	0.29	0.00284
cea2.p.119310	F58E6.1a		3.11	0.32	0.00345
cea2.i.36521	F14F8.8		3.09	0.70	0.01677
cea2.p.67860	K04C2.5		3.09	0.97	0.03161
cea2.c.28925	F49E11.10	scl-2	3.08	0.16	0.00094
cea2.p.43895	R03C1.2		3.07	1.87	0.10487
cea2.c.15098	Y38E10A.15		3.05	0.62	0.01362
cea2.d.41903	Y105C5A.4	abu-5	3.03	1.19	0.04800
cea2.i.10656	F07F6.5	dct-5	3.03	1.04	0.03743
cea2.d.08299	AC3.3	abu-1	3.01	0.80	0.02263
cea2.d.10235	C03A7.4	pqn-5	3.00	1.43	0.06811
cea2.d.48550	ZK180.5b		2.99	1.18	0.04814
cea2.i.07343	Y95B8A.1	nas-30	2.97	1.18	0.04856
cea2.p.41525	F59B10.3		2.97	1.35	0.06204
cea2.p.89456	D2096.6		2.97	1.32	0.06052
cea2.p.109736	C26F1.2	cyp-32A1	2.96	1.02	0.03729
cea2.p.126569	W03F9.4		2.96	0.74	0.01999
cea2.p.158110	T06E4.9		2.96	1.49	0.07531
cea2.d.00092	B0238.12		2.95	0.41	0.00643
cea2.d.47046	Y73F8A.9	pqn-91	2.95	0.86	0.02721
cea2.p.158399	R12E2.14		2.94	1.19	0.05037
cea2.i.06712	Y65B4BL.6		2.93	1.33	0.06240
cea2.d.65625	R12E2.15		2.93	1.42	0.07007
cea2.p.122633	R13H4.3		2.93	0.06	0.00013
cea2.p.54652	B0284.2		2.93	0.99	0.03586
cea2.3.21412	R07C12.1		2.93	0.64	0.01568
cea2.c.13679	R05H10.1		2.92	1.01	0.03731
cea2.c.33505	C02E7.6		2.92	1.23	0.05417
cea2.d.38988	T16G1.7		2.92	1.00	0.03696
cea2.c.31535	W03F8.6		2.91	1.14	0.04736
cea2.i.34107	C33G8.2		2.91	1.66	0.09385
cea2.i.32295	C02E7.10		2.91	1.49	0.07738
cea2.c.36290	F41F3.3		2.89	1.16	0.04959
cea2.p.128967	Y47D7A.13		2.87	1.16	0.05063
cea2.c.13879	T01D1.6	abu-11	2.87	1.65	0.09473
cea2.c.20582	H10E21.4		2.86	1.57	0.08738
cea2.c.17503	C23G10.11		2.86	1.73	0.10334
cea2.c.39838	Y51A2D.11	ttr-26	2.85	1.02	0.04001
cea2.i.18053	ZK666.6	clec-60	2.85	0.03	0.00003
cea2.i.26215	F45E4.5		2.85	1.55	0.08609
cea2.i.44912	T20D4.12		2.84	0.48	0.00921
cea2.3.03031	C14A6.2		2.84	0.47	0.00890
cea2.p.98912	T21D12.7		2.84	0.51	0.01072
cea2.p.55655	BE10.2		2.82	0.92	0.03327
cea2.d.04008	F55G11.4		2.82	0.49	0.00981
cea2.i.16055	W01C9.2		2.82	0.09	0.00036
cea2.i.51118	C28G1.2		2.81	0.60	0.01497
cea2.c.38944	T28H10.3		2.81	0.13	0.00068
cea2.p.39714	F44F4.4	ptr-8	2.80	0.82	0.02720
cea2.3.20798	M162.5		2.80	0.76	0.02398
cea2.3.31781	ZK682.5		2.78	1.61	0.09583
cea2.i.59645	Y105C5A.9		2.78	1.57	0.09163
cea2.i.39722	F53F1.6		2.77	0.54	0.01259
cea2.d.40595	T28A11.3		2.76	1.66	0.10301
cea2.p.115750	F32D8.7		2.75	0.85	0.03074
cea2.i.56563	R11.2		2.75	0.85	0.03063
cea2.d.08100	Y41E3.2	dpy-4	2.75	1.43	0.07989

cea2.i.38176	F32D8.3		2.73	1.29	0.06643
cea2.p.116658	F41E6.11		2.73	1.78	0.11713
cea2.d.08156	ZK180.5a		2.73	1.41	0.07847
cea2.c.28966	F53B2.8		2.73	0.67	0.01960
cea2.p.107640	C03G6.3	srj-14	2.72	1.08	0.04844
cea2.i.11566	F26G1.2		2.71	0.52	0.01205
cea2.c.27968	F30B5.1	dpy-13	2.70	1.18	0.05810
cea2.p.147112	F54F7.3		2.70	1.06	0.04788
cea2.c.35219	F15B9.8		2.68	1.06	0.04834
cea2.c.39012	W02D7.11		2.68	0.11	0.00058
cea2.p.127661	Y32G9B.1		2.68	0.66	0.01989
cea2.p.56525	C06E8.5		2.67	1.14	0.05592
cea2.c.36040	F35E12.9		2.66	1.17	0.05855
cea2.p.127399	Y6E2A.4		2.66	0.48	0.01055
cea2.c.44606	F38B6.5		2.66	1.16	0.05810
cea2.p.20022	Y18H1A.9		2.66	1.35	0.07678
cea2.d.07740	C05E4.9a	gei-7	2.66	0.75	0.02556
cea2.i.24771	C50F7.2	clx-1	2.65	0.88	0.03506
cea2.p.77723	Y47D3B.6		2.63	1.80	0.12613
cea2.p.32382	C34C6.7		2.62	1.08	0.05216
cea2.c.39795	Y47D7A.5	grl-5	2.62	0.62	0.01832
cea2.i.13394	F58G1.7		2.61	0.95	0.04104
cea2.p.119593	F59A7.2		2.61	0.60	0.01731
cea2.p.100271	W09G12.7		2.61	0.98	0.04363
cea2.p.134065	C15H9.1	nnt-1	2.60	1.65	0.11222
cea2.p.47104	T14D7.2	oac-46	2.60	0.13	0.00082
cea2.d.19988	F11G11.12	col-73	2.60	1.44	0.08858
cea2.p.61148	F09F7.6		2.60	0.16	0.00127
cea2.c.31425	T28C6.2		2.58	0.65	0.02042
cea2.d.10889	C05E4.9b	gei-7	2.57	1.09	0.05514
cea2.i.30418	Y51H4A.5		2.57	0.55	0.01490
cea2.c.43344	F11C1.6		2.57	0.57	0.01626
cea2.p.131076	ZC443.3		2.57	0.66	0.02154
cea2.c.36514	F53F1.5		2.56	0.94	0.04208
cea2.p.144770	F46C8.2	col-174	2.56	1.10	0.05661
cea2.p.104136	Y73B6BL.1		2.56	0.33	0.00547
cea2.3.08827	F10D11.6		2.55	1.28	0.07448
cea2.p.153198	T05A10.3	ttr-14	2.55	0.61	0.01874
cea2.c.34782	C52E4.1	cpr-1	2.55	0.70	0.02395
cea2.d.40878	W03D2.1a	pqn-75	2.54	1.67	0.11856
cea2.c.47097	M60.2		2.53	0.67	0.02228
cea2.i.20243	M04D8.3	ins-23	2.52	0.48	0.01206
cea2.p.107596	C02A12.4	lys-7	2.52	0.06	0.00022
cea2.p.51087	Y49F6B.10	col-71	2.51	0.99	0.04766
cea2.p.34685	E01G4.6		2.51	0.42	0.00907
cea2.p.98695	T14A8.2		2.51	0.24	0.00304
cea2.d.37260	T05G5.12	dao-6	2.50	1.51	0.10322
cea2.d.06248	W03D2.7		2.50	0.33	0.00577
cea2.p.124789	T16G1.4		2.50	1.89	0.14946
cea2.d.48547	ZK180.5b		2.50	1.45	0.09687
cea2.d.38977	T16G1.7		2.49	0.58	0.01783
cea2.p.148515	H13N06.2		2.49	1.33	0.08368
cea2.3.15198	F48G7.12		2.49	1.03	0.05234
cea2.p.112183	F08F3.7	cyp-14A5	2.48	1.55	0.10969
cea2.c.44881	F41E7.4	fip-5	2.47	1.62	0.11815
cea2.c.37261	K08H10.1	lea-1	2.47	0.66	0.02323
cea2.p.44692	R07G3.2	lips-17	2.47	0.51	0.01404
cea2.i.27100	K01A6.4		2.46	0.57	0.01749
cea2.d.49830	ZK867.1a	syd-9	2.45	0.91	0.04283

cea2.c.09546	C06A8.3		2.45	2.58	0.24149
cea2.c.36356	F45D3.4		2.45	0.85	0.03774
cea2.p.40149	F48A11.1	chs-2	2.45	2.07	0.17696
cea2.i.45948	W08A12.4		2.44	0.75	0.03025
cea2.d.10890	C05E4.9b	gei-7	2.44	0.85	0.03805
cea2.p.46322	T06D8.10		2.44	0.69	0.02594
cea2.p.32628	C38C6.3		2.44	2.02	0.17124
cea2.p.130005	Y75B7AR.1		2.44	0.48	0.01246
cea2.c.42965	F08F1.8	tth-1	2.44	0.61	0.02018
cea2.i.66956	ZK1240.4		2.43	1.62	0.12128
cea2.p.151418	R07B1.2	lec-7	2.43	0.59	0.01929
cea2.c.47604	T08A9.2	ttr-30	2.43	0.99	0.05129
cea2.p.70959	R13A5.3	ttr-32	2.42	1.27	0.08129
cea2.i.00526	C24G7.2		2.42	0.92	0.04523
cea2.i.43458	T03D8.4	grl-14	2.41	0.10	0.00053
cea2.i.08776	C08E3.5	fbxa-162	2.40	0.39	0.00851
cea2.p.51674	Y53F4B.27		2.40	1.54	0.11401
cea2.p.112336	F09G2.3		2.40	1.70	0.13450
cea2.p.11132	F52A8.3		2.39	0.52	0.01555
cea2.p.115349	F28G4.1	cyp-37B1	2.39	1.06	0.05984
cea2.p.72574	T05D4.4	osm-7	2.38	0.36	0.00732
cea2.c.32643	Y77E11A.14		2.38	0.09	0.00051
cea2.i.22790	ZK1010.4		2.38	0.88	0.04253
cea2.c.31899	Y43C5A.3		2.37	2.35	0.22200
cea2.i.01943	F25H5.8		2.37	0.17	0.00174
cea2.c.41917	C34E7.4		2.35	1.53	0.11637
cea2.i.28654	T28C6.1	grsp-2	2.35	0.55	0.01791
cea2.p.125096	T19A5.3		2.35	1.44	0.10637
cea2.p.113267	F16B4.7		2.35	2.42	0.23502
cea2.p.106721	B0024.4		2.35	1.46	0.10794
cea2.i.53060	F21E9.3	ttr-37	2.34	0.44	0.01145
cea2.d.22107	F25H8.5a	dur-1	2.34	0.84	0.04075
cea2.i.25633	F28E10.2		2.34	0.45	0.01213
cea2.d.10230	C03A7.14	abu-8	2.33	1.49	0.11395
cea2.p.150597	M03F4.6		2.33	0.74	0.03221
cea2.c.37607	R02F11.1		2.31	1.33	0.09518
cea2.i.27476	M01H9.1		2.31	0.85	0.04189
cea2.p.33276	C50D2.1		2.31	1.59	0.12882
cea2.p.137532	C45B2.7	ptr-4	2.29	0.86	0.04350
cea2.d.13084	C17B7.3		2.29	1.40	0.10477
cea2.i.42391	R07B7.12		2.29	1.14	0.07370
cea2.c.38631	T19C4.7	nlp-33	2.28	0.30	0.00580
cea2.p.15623	R11A5.7		2.28	0.58	0.02057
cea2.p.19216	W04A8.4		2.28	0.17	0.00192
cea2.d.45997	Y60A9.2		2.28	1.27	0.09006
cea2.i.54547	F53H4.3		2.27	0.22	0.00322
cea2.i.00636	C30F12.6		2.27	0.94	0.05333
cea2.d.07720	B0213.4	nlp-29	2.26	0.40	0.01003
cea2.i.49548	B0310.6		2.26	1.68	0.14505
cea2.p.96485	R02D3.6	grl-19	2.26	0.90	0.04887
cea2.i.14853	T05A7.1		2.26	0.84	0.04293
cea2.c.07850	Y65B4BR.6		2.25	1.06	0.06598
cea2.p.121813	M162.5		2.25	0.58	0.02152
cea2.i.47341	Y47D7A.15		2.25	1.07	0.06793
cea2.c.18773	F22B7.9		2.24	0.11	0.00077
cea2.c.16346	B0412.4	rps-29	2.24	2.46	0.25547
cea2.i.47220	Y46H3A.4		2.23	0.13	0.00106
cea2.i.30353	Y4C6B.2		2.23	0.42	0.01139
cea2.i.52887	F18G5.2	pes-8	2.22	0.68	0.03016

cea2.p.106941	B0222.3		2.22	1.06	0.06803
cea2.i.14710	R11F4.3		2.22	0.62	0.02529
cea2.d.10899	C05E7.1a		2.22	0.46	0.01409
cea2.p.109720	C26F1.1		2.21	0.53	0.01853
cea2.p.112063	F02D8.4		2.20	0.52	0.01786
cea2.i.44373	T10H4.11	cyp-34A2	2.20	0.70	0.03248
cea2.i.27195	K02D7.5		2.20	0.26	0.00466
cea2.p.99749	W02A2.3	pqn-74	2.20	1.40	0.11228
cea2.i.40718	H23N18.5		2.19	1.37	0.10930
cea2.d.02199	F11G11.10	col-17	2.19	1.00	0.06285
cea2.d.40596	T28A11.5		2.18	1.04	0.06845
cea2.p.38017	F29C12.1		2.17	0.89	0.05127
cea2.p.91466	F29B9.9	col-111	2.17	0.28	0.00540
cea2.c.42930	F08B12.4		2.16	0.20	0.00279
cea2.p.16402	T05E7.4		2.15	0.24	0.00418
cea2.i.59743	Y56A3A.9		2.15	1.29	0.10206
cea2.i.49176	ZK6.5	nhr-254	2.15	1.03	0.06831
cea2.i.36235	F10G2.3	clec-7	2.14	0.14	0.00146
cea2.i.45508	T28A11.4		2.14	0.77	0.04075
cea2.p.126421	W01F3.3	mlt-11	2.13	0.34	0.00850
cea2.p.32802	C41D7.2	ptr-3	2.13	1.00	0.06596
cea2.p.45954	T05C12.10	qua-1	2.12	0.49	0.01693
cea2.i.58427	ZC506.4		2.12	1.12	0.08228
cea2.d.40591	T28A11.3		2.12	0.56	0.02282
cea2.p.91251	F26D12.1		2.10	0.42	0.01277
cea2.d.22132	F25H8.5c	dur-1	2.10	0.65	0.03020
cea2.p.50120	Y46G5A.11		2.10	0.44	0.01430
cea2.p.114247	F21H7.3		2.09	0.59	0.02510
cea2.i.19244	EGAP1.1		2.09	1.01	0.06909
cea2.c.50239	F20B6.9		2.09	1.59	0.15126
cea2.i.51554	C40C9.3		2.08	0.60	0.02640
cea2.p.107932	C06B3.6		2.08	0.55	0.02238
cea2.i.57570	T23F2.4		2.08	0.88	0.05492
cea2.i.35400	C55A1.6		2.07	0.23	0.00416
cea2.c.38150	T05B4.3	phat-4	2.07	0.88	0.05456
cea2.c.25910	B0564.3		2.07	1.14	0.08821
cea2.p.94728	H06H21.10		2.07	0.71	0.03664
cea2.i.56403	R07E3.2		2.06	0.88	0.05618
cea2.c.08781	ZK909.3		2.06	0.76	0.04253
cea2.i.31586	ZK1251.2	ins-7	2.06	0.57	0.02488
cea2.c.37541	K11G9.6	mtl-1	2.05	0.96	0.06580
cea2.p.63687	F37A8.5		2.05	0.07	0.00036
cea2.p.124106	T07F10.6		2.05	0.12	0.00117
cea2.i.31956	B0222.4	tag-38	2.05	0.64	0.03084
cea2.i.28763	W02A2.5		2.04	0.41	0.01313
cea2.p.95026	JC8.8	ttr-51	2.04	0.10	0.00078
cea2.c.41613	C18A11.1		2.04	0.67	0.03405
cea2.p.52983	ZK20.1		2.03	0.43	0.01468
cea2.p.158077	F28D1.5	thn-2	2.03	1.02	0.07454
cea2.p.64947	F45H7.4	prk-2	2.03	0.11	0.00102
cea2.3.25092	T26F2.2		2.02	0.67	0.03475
cea2.p.137783	C52B9.2		2.01	0.03	0.00008
cea2.d.13083	C17B7.3		2.01	0.87	0.05689
cea2.d.44466	Y48A6B.8		2.01	0.94	0.06605
cea2.p.84024	B0218.2		2.00	0.24	0.00485
cea2.d.40551	T28A11.18		2.00	0.39	0.01238
cea2.p.33320	C50D2.7		1.99	0.92	0.06487
cea2.c.08961	B0491.2	sqt-1	1.98	0.34	0.00950
cea2.p.61872	F17C8.2	col-89	1.98	0.49	0.01988

cea2.p.145661	F48C5.1		1.98	0.15	0.00179
cea2.p.91338	F28D1.3	thn-1	1.97	0.79	0.05005
cea2.i.29352	Y11D7A.9		1.97	1.05	0.08357
cea2.i.47862	Y60C6A.1		1.95	0.39	0.01336
cea2.p.95467	K08C7.2	fmo-1	1.95	0.25	0.00538
cea2.p.135278	C29F7.2		1.95	0.54	0.02440
cea2.3.29112	Y51A2D.13a		1.95	0.20	0.00364
cea2.3.24554	T19E7.2b	skn-1	1.95	0.90	0.06464
cea2.d.13135	C17B7.9		1.95	0.20	0.00351
cea2.3.20788	M162.5		1.94	0.33	0.00954
cea2.p.114208	F21C10.11		1.94	0.31	0.00842
cea2.i.42660	R09B5.2	cnc-1	1.93	0.99	0.07767
cea2.i.12806	F49E12.10		1.93	0.15	0.00208
cea2.i.21709	Y39A3A.4		1.92	0.90	0.06570
cea2.p.114892	F25G6.6	nrs-2	1.92	0.78	0.05021
cea2.c.18694	F10E9.12		1.92	0.33	0.00992
cea2.p.138405	C56E10.3		1.92	1.51	0.15864
cea2.p.111856	D1086.2		1.92	1.26	0.11806
cea2.p.56004	C05B5.6	fbxa-155	1.92	0.82	0.05566
cea2.d.46470	Y69H2.3d		1.91	0.41	0.01489
cea2.i.09372	C18H9.6		1.91	0.18	0.00279
cea2.p.127447	Y22F5A.6	lys-3	1.91	0.29	0.00783
cea2.i.46149	Y113G7B.12		1.91	0.77	0.04970
cea2.i.35750	EGAP9.2	fut-2	1.91	0.38	0.01305
cea2.d.10909	C05E7.1b		1.90	0.10	0.00086
cea2.c.34597	C50B8.4		1.90	0.32	0.00964
cea2.c.06383	T28B8.1		1.89	0.81	0.05588
cea2.i.55536	K05B2.4		1.89	0.11	0.00117
cea2.c.12165	F40F8.7	pqm-1	1.88	0.12	0.00125
cea2.c.38698	T21C9.8	ttr-23	1.88	0.44	0.01811
cea2.p.76278	Y39A1A.19	fmo-3	1.88	0.60	0.03276
cea2.d.49805	ZK822.3c	nhx-9	1.88	0.23	0.00510
cea2.i.50105	C07A4.3		1.87	0.08	0.00056
cea2.d.24196	F36H5.2b	math-27	1.87	1.81	0.21528
cea2.i.38237	F35B12.4		1.87	0.78	0.05384
cea2.i.36420	F13A7.11		1.87	0.20	0.00387
cea2.p.121451	K11G9.5		1.87	0.38	0.01337
cea2.p.158055	C06B8.2a		1.87	0.33	0.01005
cea2.i.27611	M7.12		1.87	1.15	0.10635
cea2.c.50255	Y48G8AL.12		1.86	0.95	0.07706
cea2.p.155464	T27A10.6		1.86	0.35	0.01173
cea2.i.04332	T06G6.6		1.86	0.92	0.07343
cea2.d.41881	Y105C5A.12		1.86	0.47	0.02095
cea2.c.35222	F16B4.4		1.85	1.47	0.16035
cea2.i.00801	C36B1.6		1.85	0.04	0.00017
cea2.i.07840	B0034.1		1.84	0.25	0.00615
cea2.c.36452	F47C10.2	btb-21	1.84	0.25	0.00629
cea2.i.10574	F01D5.10		1.84	0.45	0.01957
cea2.p.129486	Y58A7A.3		1.83	0.91	0.07373
cea2.p.96611	R07C12.2		1.83	1.00	0.08712
cea2.p.07624	F22D6.10	col-60	1.82	1.06	0.09748
cea2.p.74010	T17H7.7		1.82	1.09	0.10080
cea2.i.36870	F17C11.5	clcc-221	1.82	0.75	0.05271
cea2.d.40557	T28A11.19		1.82	1.10	0.10313
cea2.p.127610	Y32F6B.1		1.82	0.13	0.00172
cea2.p.79019	Y55B1BL.1		1.81	0.20	0.00414
cea2.p.139184	F08B12.1		1.81	0.09	0.00075
cea2.d.39349	T20D4.11		1.80	0.95	0.08081
cea2.i.22476	Y75B8A.20	grl-15	1.80	0.47	0.02215

cea2.i.55411	K03A1.4		1.80	0.99	0.08742
cea2.i.17407	Y57A10A.14		1.80	0.20	0.00427
cea2.c.32012	Y54G2A.11		1.80	0.79	0.05919
cea2.p.91835	F33D4.6b		1.80	0.45	0.02014
cea2.c.09664	C07E3.3		1.80	0.49	0.02371
cea2.i.39273	F46B6.8		1.79	0.24	0.00613
cea2.p.151152	R03G8.3		1.79	0.50	0.02532
cea2.p.108682	C14C6.5		1.78	0.32	0.01059
cea2.p.146951	F53H4.2		1.78	0.83	0.06491
cea2.i.11941	F35C11.6		1.78	0.03	0.00010
cea2.p.100605	Y37A1A.3		1.78	0.17	0.00316
cea2.i.30731	Y57G11B.2		1.78	0.25	0.00669
cea2.p.56570	C06G4.5		1.78	0.57	0.03281
cea2.i.41604	K10C9.1		1.77	0.87	0.07186
cea2.i.58329	ZC373.6	dao-4	1.77	0.52	0.02796
cea2.p.31420	C25H3.10		1.77	0.46	0.02176
cea2.p.119271	F58B4.1		1.77	1.05	0.10013
cea2.c.41871	C30G4.7		1.76	0.21	0.00454
cea2.d.44779	Y51A2D.13b		1.76	0.11	0.00121
cea2.p.143080	F31F6.5	daf-6	1.76	0.46	0.02171
cea2.p.41539	F59B10.4		1.76	1.29	0.14184
cea2.p.103849	Y69A2AR.5		1.75	0.63	0.04032
cea2.p.89320	C55F2.2	ilys-4	1.75	0.39	0.01580
cea2.p.94275	F56D5.6		1.75	0.14	0.00200
cea2.i.56087	M60.7		1.75	0.61	0.03888
cea2.i.59795	Y73B6BL.15		1.74	0.46	0.02245
cea2.p.144823	F46C8.8		1.74	0.93	0.08293
cea2.i.40051	F56H9.4	gpa-9	1.74	0.93	0.08401
cea2.d.40746	W01B11.4a		1.74	0.30	0.01007
cea2.p.87417	C34D4.9	nas-8	1.73	0.78	0.06162
cea2.3.29371	Y54F10AM.2b	feh-1	1.73	0.79	0.06264
cea2.p.122425	R10E8.8		1.73	1.23	0.13501
cea2.p.76764	Y39E4B.4	tsp-3	1.73	0.47	0.02337
cea2.p.148226	F59F3.4		1.73	1.22	0.13377
cea2.c.12179	F41C3.2		1.72	0.67	0.04667
cea2.p.59625	C45G9.6a		1.72	0.18	0.00343
cea2.i.27468	K11H12.6		1.72	0.41	0.01858
cea2.i.50083	C07A12.2		1.72	0.12	0.00163
cea2.c.07327	Y40B1B.7		1.72	0.83	0.06935
cea2.c.42473	C52B9.11		1.71	0.41	0.01886
cea2.3.03206	C17B7.10		1.71	0.84	0.07195
cea2.i.25363	F17E9.4		1.71	1.44	0.17601
cea2.c.11022	F09E5.14		1.70	0.29	0.00951
cea2.p.76484	Y39A3A.3		1.70	0.10	0.00122
cea2.p.67992	K04H4.2a		1.70	1.00	0.09920
cea2.p.106931	B0213.15		1.70	0.52	0.02998
cea2.p.43390	M03A1.3		1.70	0.20	0.00468
cea2.p.107125	B0331.2		1.70	0.94	0.08831
cea2.p.90272	F11E6.8		1.70	0.44	0.02152
cea2.i.25360	F17E9.11	lys-10	1.69	0.82	0.06953
cea2.i.36844	F16B4.9	nhr-178	1.69	1.25	0.14319
cea2.p.127382	Y5H2B.5	cyp-32B1	1.69	0.81	0.06802
cea2.i.45627	W01A11.7		1.69	0.55	0.03341
cea2.c.44883	F41E7.8	cnc-8	1.68	0.64	0.04540
cea2.c.40549	ZK836.1	lon-3	1.68	0.85	0.07629
cea2.d.40559	T28A11.19		1.67	0.96	0.09489
cea2.p.106242	ZK792.4		1.67	0.21	0.00505
cea2.d.04685	M01A8.1		1.67	0.48	0.02583
cea2.p.125895	T25F10.3		1.67	0.35	0.01398

cea2.p.158153	C13B9.1		1.67	1.32	0.15982
cea2.i.29324	Y11D7A.5		1.67	0.54	0.03367
cea2.d.43845	Y43F8B.7		1.67	0.66	0.04808
cea2.i.23814	C28C12.4		1.67	0.63	0.04424
cea2.c.30574	R05G6.10		1.67	0.21	0.00505
cea2.i.42937	R12G8.1		1.67	0.73	0.05834
cea2.3.13631	F38B7.1a	ccch-1	1.66	0.57	0.03710
cea2.d.02230	F13B10.1b	tir-1	1.66	0.94	0.09120
cea2.c.13465	K12H6.3	fut-4	1.66	0.51	0.03023
cea2.i.32486	C03G6.5		1.66	0.63	0.04489
cea2.p.137146	C42D8.5		1.66	0.46	0.02439
cea2.i.43129	R90.4	ttr-29	1.66	0.65	0.04807
cea2.p.155603	T28B4.4		1.65	0.16	0.00315
cea2.i.19219	E02H9.4		1.65	0.82	0.07406
cea2.p.91376	F28D1.9		1.64	0.83	0.07591
cea2.i.47371	Y49A3A.4		1.64	1.50	0.19889
cea2.c.47627	T08A9.12	spp-2	1.64	0.63	0.04522
cea2.c.48369	W05H9.1		1.64	0.34	0.01424
cea2.p.150820	M163.1		1.64	0.26	0.00844
cea2.p.28137	B0286.2		1.64	0.27	0.00916
cea2.d.11026	C06B8.2b		1.63	0.77	0.06731
cea2.p.87866	C42D4.1		1.62	0.20	0.00504
cea2.p.119636	F59B1.8		1.62	0.14	0.00238
cea2.p.98467	T12G3.1		1.62	0.24	0.00695
cea2.d.40578	T28A11.20		1.62	1.03	0.11207
cea2.p.39893	F45E10.2		1.61	0.51	0.03174
cea2.p.102053	Y51H4A.8		1.61	0.49	0.02940
cea2.i.26632	F56H11.2		1.61	0.09	0.00100
cea2.c.10969	F08B1.1a	vhp-1	1.61	0.57	0.03955
cea2.p.101702	Y43E12A.2		1.61	0.74	0.06469
cea2.3.19633	K08D9.4		1.60	0.96	0.10186
cea2.p.31217	C23H3.7	tre-5	1.60	0.57	0.04001
cea2.c.10393	C32D5.9	lgg-1	1.60	0.23	0.00672
cea2.3.12362	F28F5.3c	lim-8	1.59	0.18	0.00408
cea2.p.56296	C05D11.6	nas-4	1.59	0.47	0.02733
cea2.p.91965	F36A4.8	ttr-20	1.59	0.25	0.00816
cea2.3.16616	F55G11.7		1.59	0.44	0.02429
cea2.i.18285	ZK970.6	gcy-5	1.59	0.72	0.06258
cea2.i.30269	Y46C8AL.2	clec-174	1.59	0.54	0.03647
cea2.p.77872	Y48A6B.9		1.58	0.64	0.05091
cea2.d.27928	F54D10.2	fbxa-24	1.58	0.15	0.00310
cea2.i.55212	H03E18.2		1.58	0.20	0.00527
cea2.3.29824	Y58A7A.4		1.58	1.14	0.13824
cea2.i.35868	F07C3.9		1.58	0.40	0.02065
cea2.p.114053	F21A3.2		1.58	0.03	0.00014
cea2.i.09325	C18E9.1	cal-2	1.57	0.66	0.05452
cea2.i.10781	F09C12.1	ggr-3	1.57	0.83	0.08131
cea2.i.26874	H04M03.2	nspb-6	1.57	0.58	0.04216
cea2.d.41218	W06B3.2b	sma-5	1.57	0.06	0.00047
cea2.i.18601	C07A9.1	clec-162	1.57	0.60	0.04507
cea2.i.33264	C13G3.1		1.56	0.31	0.01254
cea2.d.46965	Y73C8B.1		1.56	0.95	0.10465
cea2.i.13065	F54D10.8		1.56	0.28	0.01089
cea2.p.154133	T14F9.4		1.56	0.44	0.02606
cea2.p.04877	C45E1.4		1.56	1.08	0.12904
cea2.p.113487	F17C11.11		1.55	1.07	0.12923
cea2.p.98365	T12A7.6		1.55	0.60	0.04686
cea2.p.70603	R10E11.9		1.55	0.45	0.02680
cea2.d.11175	C06H5.2	fbxa-157	1.55	0.28	0.01072

cea2.d.39995	T24A6.16		1.54	0.58	0.04444
cea2.p.129914	Y69H2.10		1.54	0.74	0.06978
cea2.p.124265	T08B1.6		1.54	0.24	0.00807
cea2.d.21840	F23H11.8a	pef-1	1.54	0.88	0.09403
cea2.c.33673	C06C6.4	nhr-63	1.54	0.20	0.00567
cea2.i.10513	E04F6.8		1.54	0.75	0.07125
cea2.c.10902	F01D5.5		1.54	0.45	0.02765
cea2.p.23179	Y65B4A.2		1.53	0.49	0.03291
cea2.p.122854	T01C2.1	acy-4	1.53	0.37	0.01922
cea2.c.34372	C37C3.13	ttr-33	1.53	0.51	0.03548
cea2.p.11314	F53B6.1	tsp-15	1.53	0.62	0.05108
cea2.p.55680	C02C2.1	tyr-1	1.53	0.41	0.02274
cea2.d.41601	W09D6.1a		1.53	0.77	0.07440
cea2.i.00182	C01H6.1	col-61	1.53	0.60	0.04830
cea2.i.31672	ZK593.9		1.53	0.68	0.05952
cea2.d.21445	F20C5.2b	klp-11	1.53	1.20	0.15910
cea2.i.24934	C55C3.1		1.52	0.83	0.08568
cea2.i.22724	ZC395.5		1.52	0.40	0.02201
cea2.p.158146	B0564.6		1.52	0.29	0.01147
cea2.c.13291	K02C4.4	ltd-1	1.52	0.61	0.05043
cea2.i.39288	F47B8.2		1.52	0.39	0.02136
cea2.i.37076	F20D6.10		1.51	0.86	0.09310
cea2.p.142091	F22E10.3	pgp-14	1.51	0.56	0.04285
cea2.c.40464	ZC513.12	sth-1	1.51	0.72	0.06742
cea2.p.152365	T01C1.4		1.51	0.82	0.08578
cea2.d.18172	D1054.9a		1.51	0.72	0.06785
cea2.i.26047	F42A9.4	cyp-33E3	1.51	0.68	0.06069
cea2.i.27631	M7.8		1.50	0.75	0.07353
cea2.d.01994	F02A9.3	far-2	1.50	1.08	0.13858
cea2.d.46971	Y73C8B.1		1.50	0.59	0.04711
cea2.i.41972	M02H5.3	nhr-201	1.50	0.99	0.11931
cea2.c.38847	T27F2.4		1.50	0.33	0.01549
cea2.d.08179	F53A9.2		1.50	0.16	0.00399
cea2.c.45879	F56C3.9		1.50	0.51	0.03680
cea2.c.22567	T03F6.4		1.49	0.52	0.03809
cea2.c.25013	ZC21.2		1.49	1.25	0.17524
cea2.p.112219	F09C6.1		1.49	0.43	0.02635
cea2.i.40519	F59E11.12		1.49	1.17	0.15916
cea2.i.49631	B0563.1		1.49	0.28	0.01121
cea2.d.40552	T28A11.18		1.49	0.64	0.05652
cea2.d.07904	F38B7.1b	ccch-1	1.49	0.61	0.05176
cea2.p.40227	F52C6.7		1.48	1.17	0.15843
cea2.d.07285	Y71G12B.25		1.48	0.62	0.05440
cea2.d.39354	T20D4.15		1.48	0.88	0.10057
cea2.p.120676	K07C5.7	ttl-15	1.48	1.01	0.12737
cea2.p.15075	M01G12.12	rrf-2	1.47	1.31	0.19122
cea2.p.62195	F23F12.1		1.47	0.92	0.10988
cea2.p.123593	T05E12.3		1.47	0.94	0.11278
cea2.p.47717	T24E12.5		1.47	0.27	0.01132
cea2.c.12485	F46C5.1		1.47	0.36	0.01954
cea2.p.126441	W02F12.2		1.47	0.19	0.00539
cea2.d.39346	T20D4.11		1.46	0.58	0.04852
cea2.i.50371	C11G10.1		1.45	0.50	0.03743
cea2.i.09370	C18H9.5		1.45	0.25	0.00985
cea2.p.107602	C03A7.11	ugt-51	1.45	0.21	0.00685
cea2.c.40586	ZK863.8		1.45	1.02	0.13315
cea2.p.16540	T05F1.11		1.45	1.40	0.21446
cea2.p.53800	ZK970.1		1.45	0.17	0.00472
cea2.p.109643	C25F9.1		1.45	0.56	0.04683

cea2.p.148282	F59F5.3		1.45	1.36	0.20639
cea2.p.37269	F21D12.3		1.44	0.39	0.02318
cea2.3.22102	R52.2		1.43	0.38	0.02263
cea2.p.29370	C06A8.9	glr-4	1.43	0.36	0.02052
cea2.c.48479	Y15E3A.5		1.43	0.55	0.04553
cea2.p.101406	Y41D4B.26		1.43	0.63	0.05881
cea2.p.43488	M05D6.7	gbh-2	1.43	0.36	0.02083
cea2.i.10620	F07E5.6	fbxb-36	1.43	0.93	0.11683
cea2.c.38836	T27E4.7		1.43	0.16	0.00404
cea2.p.105393	ZC410.4		1.43	0.98	0.12707
cea2.p.47606	T24B8.4		1.42	0.21	0.00716
cea2.d.07900	F36H5.2a	math-27	1.42	0.77	0.08463
cea2.i.29862	Y39C12A.1		1.42	0.36	0.02071
cea2.d.13075	C17B7.2		1.42	0.59	0.05291
cea2.d.36665	T03F7.7b		1.42	0.73	0.07846
cea2.d.07214	Y69H2.3b		1.42	0.52	0.04178
cea2.c.34167	C25E10.8		1.41	0.59	0.05314
cea2.i.45174	T24A6.3		1.41	0.59	0.05451
cea2.p.102003	Y46C8AL.5	clec-72	1.40	0.31	0.01612
cea2.p.153382	T07F12.4		1.40	0.22	0.00799
cea2.3.28746	Y48C3A.5a		1.40	0.66	0.06787
cea2.p.125294	T20B3.1		1.40	0.92	0.11994
cea2.c.43615	F14H12.3		1.40	0.46	0.03489
cea2.p.133203	C09B7.1b	ser-7	1.39	0.44	0.03100
cea2.d.04765	M03F8.2b	pst-1	1.39	1.04	0.14680
cea2.3.09026	F11A5.10	glc-1	1.39	0.72	0.07812
cea2.i.04493	T09E11.11		1.39	0.60	0.05757
cea2.c.48067	T20B5.2		1.39	0.30	0.01550
cea2.i.13260	F58A6.1		1.39	0.45	0.03367
cea2.d.06332	W06B3.2a	sma-5	1.39	0.11	0.00207
cea2.i.45366	T26H5.4		1.39	0.60	0.05666
cea2.i.04437	T08G11.3		1.39	0.39	0.02558
cea2.p.112937	F13H6.1		1.38	0.56	0.05019
cea2.i.35318	C54F6.4		1.38	0.65	0.06771
cea2.p.128505	Y43F8C.3		1.37	0.39	0.02641
cea2.d.11072	C06C6.5a	nhr-50	1.37	0.18	0.00542
cea2.i.03820	K11D2.1		1.37	0.22	0.00880
cea2.p.121608	M02H5.1	nhr-99	1.37	0.89	0.11643
cea2.p.33446	C52E2.4		1.37	0.48	0.03941
cea2.p.102061	Y51H4A.9	col-137	1.37	0.77	0.09094
cea2.d.46074	Y61A9LA.4		1.36	0.22	0.00853
cea2.3.30096	Y69H2.3b		1.36	0.48	0.03838
cea2.p.90417	F13B6.3		1.36	0.14	0.00340
cea2.p.103972	Y69A2AR.19		1.36	0.40	0.02705
cea2.i.52215	E03G2.4	col-186	1.36	0.72	0.08134
cea2.i.05346	W05F2.5	fbxa-203	1.36	1.00	0.14373
cea2.i.58081	Y62H9A.12		1.35	0.57	0.05354
cea2.c.21157	K02F3.4	zip-2	1.35	0.22	0.00834
cea2.c.40144	Y70C5C.6		1.35	0.54	0.04948
cea2.d.45414	Y54G11A.5b		1.35	0.60	0.05937
cea2.p.156299	Y34B4A.10		1.35	0.04	0.00030
cea2.p.30332	C15F1.2		1.35	0.43	0.03226
cea2.p.12528	F56C11.6		1.34	0.09	0.00150
cea2.c.16485	C02F5.8	tsp-1	1.34	0.63	0.06645
cea2.c.01566	C50F2.10	abf-2	1.34	0.05	0.00043
cea2.d.42775	Y19D10A.7		1.34	0.88	0.11790
cea2.p.158371	ZC47.10		1.34	1.11	0.17315
cea2.c.35812	F28F8.2	acs-2	1.34	0.57	0.05485
cea2.3.12325	F28F5.3b	lim-8	1.33	0.28	0.01425

cea2.i.43353	T02B11.4		1.33	0.11	0.00235
cea2.c.31629	Y5F2A.2	ttr-17	1.32	0.76	0.09452
cea2.p.137333	C44E12.1		1.32	0.61	0.06507
cea2.p.04178	C37A5.1		1.32	1.03	0.15640
cea2.3.12512	F29G9.4		1.32	0.37	0.02485
cea2.i.56311	R04B3.3		1.32	0.56	0.05493
cea2.p.131125	ZC455.1		1.32	0.35	0.02305
cea2.i.25299	F13H10.1		1.32	0.21	0.00817
cea2.p.66755	F58B6.2	inf-1	1.31	0.03	0.00017
cea2.i.12664	F45E12.6		1.31	0.39	0.02854
cea2.p.34660	E01G4.5		1.31	0.41	0.03152
cea2.c.32313	Y67D8B.4		1.31	0.38	0.02730
cea2.i.35212	C54D10.9		1.31	0.40	0.02947
cea2.d.46464	Y69H2.3c		1.30	0.05	0.00052
cea2.i.37985	F31D4.4	clec-264	1.30	0.81	0.10892
cea2.p.155826	W04G3.8	lpr-3	1.30	0.42	0.03385
cea2.p.100497	Y11D7A.14		1.30	0.42	0.03262
cea2.i.25235	F13B6.1		1.30	0.30	0.01735
cea2.i.59357	F35F10.13		1.30	0.38	0.02734
cea2.p.110194	C33G8.11	nhr-107	1.30	0.79	0.10416
cea2.i.47201	Y45G12C.1		1.29	0.16	0.00481
cea2.i.62758	F53A9.1		1.29	0.24	0.01147
cea2.i.52115	D1053.3		1.29	0.95	0.14335
cea2.p.110142	C31A11.5	oac-6	1.29	0.23	0.01052
cea2.d.13212	C17G10.4a	cdc-14	1.29	0.78	0.10284
cea2.d.42760	Y19D10A.5		1.29	0.39	0.02927
cea2.c.06403	T28F4.5		1.29	0.52	0.04976
cea2.p.117493	F47H4.10	skr-5	1.28	0.38	0.02746
cea2.p.138171	C54D2.4		1.28	0.99	0.15305
cea2.p.110272	C34B4.1		1.28	0.73	0.09352
cea2.p.131703	ZK1037.6		1.28	0.05	0.00058
cea2.p.85301	C05C12.4		1.28	0.88	0.12771
cea2.c.18778	F23F12.9a		1.28	0.48	0.04451
cea2.d.11551	C09B7.1a	ser-7	1.28	0.23	0.01049
cea2.p.40292	F52H3.5		1.28	0.55	0.05675
cea2.c.26608	C30H6.10		1.27	0.11	0.00255
cea2.c.29283	F58F9.7		1.27	0.16	0.00535
cea2.p.158073	F15H10.1	col-12	1.27	0.40	0.03172
cea2.p.132317	C02F12.7	tag-278	1.27	0.77	0.10400
cea2.i.53427	F34H10.2		1.27	0.78	0.10552
cea2.d.27071	F49C5.3		1.27	0.36	0.02638
cea2.i.05362	W06D4.3		1.26	0.12	0.00280
cea2.p.50410	Y48B6A.6a		1.26	0.34	0.02288
cea2.i.40586	H09F14.1		1.26	0.46	0.04236
cea2.i.57842	W03G11.2		1.26	0.03	0.00016
cea2.i.22663	Y82E9BR.4		1.26	0.64	0.07600
cea2.i.11182	F14D2.7		1.26	0.17	0.00595
cea2.p.140340	F13D11.1		1.26	0.27	0.01528
cea2.p.80475	Y75B8A.32		1.26	0.50	0.04941
cea2.p.56717	C07A9.8		1.26	0.31	0.02028
cea2.p.146397	F52B10.1	nmy-1	1.26	0.04	0.00037
cea2.p.109111	C16D9.4		1.25	0.18	0.00690
cea2.p.23315	Y65B4BR.2	lpr-1	1.25	0.48	0.04626
cea2.p.117319	F46E10.11		1.25	0.37	0.02810
cea2.p.103343	Y67A10A.6	ssu-2	1.25	0.72	0.09501
cea2.d.21290	F18E9.5b	tag-279	1.25	0.03	0.00015
cea2.i.23103	B0496.8	tag-224	1.25	0.30	0.01823
cea2.p.33418	C52A11.4		1.25	0.26	0.01412
cea2.p.128638	Y44A6C.1		1.24	0.51	0.05267

cea2.p.46961	T13H5.3		1.24	0.02	0.00005
cea2.c.06942	W10D5.1	mef-2	1.24	0.18	0.00709
cea2.i.34104	C33G8.12	nhr-163	1.24	0.48	0.04633
cea2.p.68857	K10F12.4b	gst-3	1.24	0.28	0.01686
cea2.p.03445	C34G6.2	tyr-4	1.24	0.52	0.05431
cea2.p.140325	F13D2.1		1.24	0.77	0.10750
cea2.p.48433	W02B8.5	hot-3	1.23	0.55	0.05927
cea2.p.38364	F32A11.6	moe-3	1.23	0.27	0.01547
cea2.c.13735	R10H1.4	isl-1	1.23	0.16	0.00543
cea2.c.03615	F40E3.5		1.23	0.43	0.03805
cea2.p.130260	Y97E10C.1		1.23	0.23	0.01153
cea2.d.24751	F40E3.6		1.23	0.22	0.01017
cea2.i.13594	K01A2.10		1.23	0.43	0.03915
cea2.p.48530	W03C9.6		1.23	0.09	0.00165
cea2.d.07795	C34G6.6a	noah-1	1.23	0.23	0.01180
cea2.i.57623	T24C2.5		1.23	0.41	0.03513
cea2.i.49657	B0563.7		1.23	0.06	0.00079
cea2.c.28375	F33D4.3	flp-13	1.22	0.08	0.00130
cea2.d.29671	F56H11.1b	fbl-1	1.22	0.17	0.00626
cea2.c.18280	D2045.8		1.22	0.62	0.07738
cea2.p.121092	K10D6.4		1.21	0.21	0.01018
cea2.p.46606	T09A5.1		1.21	0.24	0.01295
cea2.d.30170	F58B3.1	lys-4	1.21	0.65	0.08458
cea2.d.20462	F13E9.11		1.21	0.27	0.01631
cea2.p.89386	D1046.5		1.21	0.36	0.02837
cea2.3.06693	C48D5.2a	ptp-1	1.21	0.29	0.01909
cea2.i.32849	C08E8.4		1.21	0.57	0.06666
cea2.i.42395	R07B7.13	nhr-206	1.21	0.19	0.00840
cea2.p.145973	F48F7.2	syn-2	1.20	0.31	0.02075
cea2.i.47618	Y51A2B.1		1.20	0.18	0.00770
cea2.i.55296	H22K11.3		1.20	0.08	0.00163
cea2.i.50182	C09B8.8		1.20	0.29	0.01922
cea2.i.41994	M02H5.5	nhr-203	1.20	0.27	0.01622
cea2.p.31635	C27H5.2c		1.20	0.93	0.15383
cea2.d.25379	F42G9.9a	ptl-1	1.20	0.47	0.04743
cea2.p.158048	ZK1193.5		1.20	0.21	0.01012
cea2.p.150721	M60.6		1.20	0.18	0.00782
cea2.d.01455	C46H11.4b	lfe-2	1.19	0.19	0.00803
cea2.p.119816	H14N18.4a		1.19	0.34	0.02619
cea2.p.128466	Y43F8B.9		1.19	0.34	0.02566
cea2.p.25822	ZC123.1		1.19	0.49	0.05133
cea2.p.31602	C27H5.2a		1.19	0.56	0.06732
cea2.d.07787	C27H6.4a	rmd-2	1.19	0.12	0.00321
cea2.p.107338	B0507.1		1.18	0.29	0.01962
cea2.i.40335	F58H1.4		1.18	0.10	0.00224
cea2.3.04377	C27H5.4		1.18	0.45	0.04575
cea2.p.134456	C18B12.4		1.18	0.17	0.00708
cea2.i.42355	R07B5.3	sru-40	1.18	0.27	0.01688
cea2.c.24137	Y47D3B.10	dpy-18	1.18	0.32	0.02386
cea2.d.13067	C17B7.2		1.18	0.36	0.02898
cea2.p.149401	K06A9.2		1.18	0.36	0.03041
cea2.p.140361	F13D11.4		1.18	0.24	0.01375
cea2.p.52971	ZC204.12		1.18	0.46	0.04739
cea2.d.08144	Y69H2.3a		1.18	0.31	0.02280
cea2.d.19500	F10C1.8a		1.17	0.36	0.03045
cea2.d.44465	Y48A6B.8		1.17	0.49	0.05257
cea2.c.07132	Y18H1A.2		1.17	0.55	0.06606
cea2.d.09831	C01B10.5a	hil-7	1.17	0.25	0.01532
cea2.3.10098	F15G9.1b		1.17	0.08	0.00138

cea2.d.06884	Y51A2D.13a		1.17	0.21	0.01026
cea2.c.13265	K02B7.3		1.17	0.13	0.00419
cea2.3.18617	K01A2.3b		1.17	0.52	0.05953
cea2.d.08055	T21D12.9a		1.17	0.37	0.03157
cea2.i.11529	F22B5.6		1.17	0.04	0.00047
cea2.p.155563	T27B1.2	ztf-19	1.17	0.21	0.01050
cea2.p.145546	F47E1.1		1.17	0.11	0.00272
cea2.d.10726	C05D10.4c		1.16	0.03	0.00016
cea2.c.46997	K11G12.7	acr-3	1.16	0.48	0.05257
cea2.p.123294	T03E6.8		1.16	0.70	0.10293
cea2.d.21313	F19C6.2b		1.16	0.35	0.02977
cea2.c.36758	F58E6.1b		1.16	0.15	0.00567
cea2.p.10649	F45H11.4	mgl-2	1.16	0.29	0.02071
cea2.3.08500	F08B1.1b	vhp-1	1.16	0.21	0.01044
cea2.i.34233	C36C5.5		1.16	0.22	0.01134
cea2.p.153566	T09B9.2		1.16	0.53	0.06297
cea2.p.150580	M03F4.3		1.15	0.20	0.01011
cea2.3.04379	C27H5.4		1.15	0.42	0.04216
cea2.p.03143	C32E12.4		1.15	0.53	0.06442
cea2.p.46201	T06D8.1a		1.15	0.23	0.01320
cea2.3.10303	F16B4.12b	nhr-117	1.15	0.48	0.05446
cea2.p.123198	T02E9.5		1.14	0.18	0.00844
cea2.i.15818	T24B8.5		1.14	0.21	0.01152
cea2.p.18011	T23G11.6		1.14	0.17	0.00740
cea2.p.80054	Y75B8A.3		1.14	0.37	0.03281
cea2.c.19391	F43D9.4	sip-1	1.14	0.24	0.01485
cea2.i.29576	Y37E11AR.4		1.14	0.20	0.00992
cea2.d.43790	Y41E3.6		1.14	0.06	0.00082
cea2.i.46315	Y17D7B.2		1.14	0.26	0.01657
cea2.p.99313	T25B9.7	ugt-54	1.14	0.46	0.05154
cea2.p.15343	R06A10.4		1.14	0.19	0.00947
cea2.p.48022	T25D10.4		1.13	0.15	0.00541
cea2.i.17881	ZK177.3		1.13	0.13	0.00405
cea2.p.56427	C06E1.3		1.13	0.24	0.01521
cea2.c.17471	C23G10.3	rps-3	1.13	0.60	0.08344
cea2.c.22743	T07C4.9		1.13	0.22	0.01265
cea2.c.09565	C06A8.11		1.13	0.63	0.09060
cea2.p.132241	C02C6.2		1.13	0.34	0.02830
cea2.i.33904	C26E1.2		1.13	0.54	0.06972
cea2.p.157664	ZK563.2		1.13	0.21	0.01098
cea2.i.14878	T05A7.7		1.12	0.60	0.08342
cea2.c.37060	K03B8.9	deg-3	1.12	0.27	0.01833
cea2.d.02964	F32A5.4b		1.12	0.63	0.09159
cea2.d.48909	ZK337.1b		1.12	0.50	0.06003
cea2.p.30301	C14A4.12		1.12	0.55	0.07127
cea2.c.50707	T23F2.3		1.12	0.29	0.02136
cea2.c.39513	Y38A10A.5	crt-1	1.12	0.40	0.03981
cea2.c.23702	Y37D8A.16		1.12	0.05	0.00076
cea2.p.156588	Y102A11A.2		1.12	0.56	0.07523
cea2.p.66240	F56D2.3		1.12	0.35	0.03099
cea2.p.134913	C25B8.6	nhr-120	1.12	0.29	0.02170
cea2.i.57281	T18D3.3		1.11	0.06	0.00087
cea2.i.08140	B0252.5		1.11	0.29	0.02224
cea2.d.39710	T22H2.5b	scrm-1	1.11	0.13	0.00442
cea2.3.08633	F08F1.1b	kin-9	1.11	0.57	0.07800
cea2.c.26820	C39E9.8		1.11	0.44	0.04889
cea2.p.121921	R02D5.3		1.11	0.34	0.02997
cea2.c.08812	B0034.3a	casy-1	1.11	0.38	0.03714
cea2.p.50514	Y48C3A.4	ztf-22	1.11	0.17	0.00814

cea2.p.119275	F58B4.3		1.11	0.18	0.00848
cea2.i.00129	B0511.5	cutl-14	1.11	0.36	0.03393
cea2.p.145396	F47A4.5		1.11	0.23	0.01447
cea2.p.131291	ZK287.1		1.10	0.69	0.11051
cea2.p.02203	C17H1.7		1.10	0.15	0.00582
cea2.c.36824	F58E10.7		1.10	0.20	0.01120
cea2.p.10954	F47G6.2		1.10	0.38	0.03687
cea2.3.28823	Y48E1B.4		1.10	0.57	0.07878
cea2.c.27633	F13H10.6		1.10	0.34	0.03048
cea2.p.143602	F38E9.1		1.10	0.52	0.06800
cea2.3.00120	B0024.14c	crm-1	1.09	0.06	0.00088
cea2.d.07788	C28C12.7a	spp-10	1.09	0.11	0.00365
cea2.i.39092	F44C4.5	ppt-1	1.09	0.36	0.03393
cea2.3.13004	F35B3.5		1.09	0.27	0.01922
cea2.p.33859	C56E6.6		1.09	0.75	0.12930
cea2.d.26987	F48E3.8b		1.08	0.58	0.08415
cea2.p.133399	C10E2.3	hda-4	1.08	0.25	0.01723
cea2.c.12310	F43G6.8		1.08	0.19	0.01010
cea2.p.52016	Y54G11B.1		1.08	0.15	0.00621
cea2.c.44096	F18G5.6		1.08	0.26	0.01813
cea2.c.39363	W06H8.5		1.08	0.10	0.00285
cea2.p.157330	ZK54.2		1.08	0.35	0.03378
cea2.d.27724	F53B2.2	tsp-4	1.07	0.44	0.05154
cea2.c.12601	F54C9.3		1.07	0.62	0.09506
cea2.p.141522	F19C6.2a		1.07	0.21	0.01252
cea2.i.43533	T04H1.1	tag-348	1.07	0.08	0.00179
cea2.p.137184	C43C3.3	dyf-8	1.07	0.18	0.00911
cea2.p.153722	T10B10.6	phat-6	1.07	0.53	0.07367
cea2.i.59268	F14D7.7		1.07	0.11	0.00368
cea2.p.107728	C05A2.1	pph-1	1.07	0.12	0.00449
cea2.3.17968	H14N18.4b		1.07	0.52	0.07013
cea2.p.158062	C08H9.7		1.07	0.28	0.02150
cea2.p.150249	K11G12.1		1.07	0.50	0.06668
cea2.c.29087	F55B11.4		1.07	0.14	0.00585
cea2.p.107455	C01B7.4	tag-117	1.06	0.68	0.11411
cea2.i.33307	C14C10.7	ttr-43	1.06	0.35	0.03526
cea2.d.11176	C06H5.2	fbxa-157	1.06	0.38	0.04064
cea2.p.05909	D2005.2	nlp-8	1.06	0.26	0.01981
cea2.3.06518	C46H11.4a	lfe-2	1.06	0.49	0.06375
cea2.p.93723	F54D1.6		1.06	0.25	0.01858
cea2.c.19930	F54E7.7	rcn-1	1.06	0.08	0.00191
cea2.i.21121	W04B5.2		1.05	0.25	0.01805
cea2.p.144790	F46C8.5	ceh-14	1.05	0.18	0.01014
cea2.p.124239	T08B1.4		1.05	0.08	0.00208
cea2.p.140369	F13E6.1		1.05	0.26	0.01989
cea2.c.37183	K07C11.5	cri-2	1.05	0.36	0.03761
cea2.f.08183	ZC239.14		1.05	0.46	0.05832
cea2.c.05228	M05B5.2		1.05	0.09	0.00253
cea2.p.36227	F13D12.8		1.05	0.38	0.04189
cea2.i.02059	F28D9.2		1.04	0.35	0.03633
cea2.c.29964	K08C7.5	fmo-2	1.04	0.37	0.04054
cea2.c.16767	C05D11.4	let-756	1.04	0.46	0.06031
cea2.p.78949	Y55B1AL.1		1.04	0.10	0.00285
cea2.c.37693	R09B5.8	cnc-3	1.04	0.36	0.03892
cea2.d.47570	ZC239.13		1.04	0.39	0.04513
cea2.p.148844	K02A6.3		1.03	0.37	0.04062
cea2.c.28749	F42C5.8	rps-8	1.03	0.27	0.02223
cea2.p.117093	F45D3.2		1.03	0.35	0.03648
cea2.3.03078	C15B12.5b	gar-1	1.03	0.61	0.09862

cea2.c.42419	C46F4.2	acs-17	1.03	0.27	0.02265
cea2.c.05145	M01D7.5	nlp-12	1.03	0.25	0.01850
cea2.p.149095	K03A11.5		1.03	0.24	0.01825
cea2.i.08192	B0286.5	fkf-6	1.03	0.38	0.04301
cea2.c.00360	C03C11.2	fog-3	1.02	0.11	0.00414
cea2.d.07439	ZC190.4		1.02	0.34	0.03562
cea2.d.14220	C25E10.9a	swm-1	1.02	0.34	0.03526
cea2.c.14445	T24H10.2		1.01	0.34	0.03496
cea2.p.34726	E02H1.7	nhr-19	1.01	0.21	0.01412
cea2.p.07559	F21F3.3		1.01	0.42	0.05216
cea2.p.89293	C55C3.5		1.01	0.26	0.02113
cea2.d.41239	W06F12.2b		1.01	0.52	0.07914
cea2.p.109717	C26E1.3		1.01	0.25	0.02006
cea2.p.11402	F53F10.1		1.01	0.25	0.01925
cea2.3.12088	F26F12.3a		1.01	0.31	0.03056
cea2.c.26642	C33A12.4		1.01	0.37	0.04175
cea2.3.26493	W06F12.2a		1.01	0.56	0.08925
cea2.p.150266	K11G12.4		1.01	0.20	0.01245
cea2.i.55373	K02D3.1		1.00	0.12	0.00469
cea2.3.19906	K10G9.1		1.00	0.28	0.02440
cea2.3.27795	Y38B5A.1		1.00	0.42	0.05469
cea2.p.91018	F21D5.3		1.00	0.50	0.07419
cea2.c.01104	C32F10.4		1.00	0.10	0.00341
cea2.i.19672	F47D12.6		1.00	0.51	0.07792
cea2.p.157027	ZC373.4		1.00	0.36	0.03999
cea2.d.29670	F56H11.1b	fbl-1	1.00	0.38	0.04481
cea2.c.39415	W08A12.2		0.99	0.19	0.01198
cea2.3.09548	F13B10.1b	tir-1	0.99	0.23	0.01741
cea2.p.97623	T04B2.5		0.99	0.41	0.05247
cea2.3.22191	T01B6.3b		0.99	0.33	0.03456
cea2.c.44966	F42G10.2	mkk-4	0.99	0.20	0.01285
cea2.p.138954	F01G12.5a	let-2	0.99	0.28	0.02507
cea2.c.19997	F55H2.1	sod-4	0.99	0.43	0.05701
cea2.i.42923	R11H6.4		0.99	0.50	0.07618
cea2.p.158384	F08F8.5	numr-1	0.98	0.38	0.04611
cea2.3.09527	F13B10.1a	tir-1	0.98	0.17	0.01029
cea2.p.55042	B0336.11a		0.98	0.53	0.08469
cea2.p.131092	ZC443.4		0.98	0.45	0.06528
cea2.c.23605	Y22D7AL.15		0.98	0.31	0.03292
cea2.i.38041	F31F4.12	nhr-180	0.97	0.17	0.00962
cea2.i.21351	Y111B2A.21		0.97	0.11	0.00445
cea2.i.26084	F42C5.2		0.97	0.37	0.04521
cea2.p.148379	H03E18.1		0.97	0.34	0.03858
cea2.p.87146	C29E6.2	trpa-1	0.97	0.48	0.07446
cea2.d.04204	F59C6.11		0.96	0.28	0.02725
cea2.p.75743	Y22D7AR.6		0.96	0.07	0.00195
cea2.i.44714	T19C4.1		0.96	0.28	0.02818
cea2.i.39484	F48G7.2		0.96	0.32	0.03624
cea2.p.128838	Y45G5AM.7		0.96	0.27	0.02549
cea2.p.145597	F47E1.4		0.95	0.27	0.02597
cea2.p.94614	H02I12.3	tag-89	0.95	0.42	0.06015
cea2.c.44782	F39C12.3a	tsp-14	0.95	0.04	0.00054
cea2.c.36079	F38A6.1		0.95	0.09	0.00283
cea2.c.13988	T05A6.1	cki-1	0.95	0.09	0.00300
cea2.3.16595	F55C7.7e	unc-73	0.95	0.10	0.00358
cea2.p.66181	F56C9.8		0.95	0.11	0.00436
cea2.p.07085	F14B6.2		0.94	0.44	0.06609
cea2.i.23302	C06E4.2		0.94	0.26	0.02468
cea2.p.56965	C09F5.2		0.94	0.19	0.01343

cea2.c.12208	F41C3.8		0.94	0.31	0.03347
cea2.p.153890	T10H10.3		0.94	0.26	0.02478
cea2.p.146292	F49H12.3		0.94	0.12	0.00505
cea2.p.102185	Y54G2A.9	clec-81	0.94	0.24	0.02134
cea2.p.26298	ZK265.7		0.94	0.17	0.01078
cea2.d.19480	F09F7.7a		0.94	0.25	0.02312
cea2.p.110183	C33G8.6	nhr-42	0.93	0.26	0.02520
cea2.i.50910	C23H4.7		0.93	0.09	0.00298
cea2.i.59356	F34H10.1		0.93	0.05	0.00084
cea2.i.00046	B0207.10		0.93	0.08	0.00268
cea2.c.14007	T05A8.3		0.93	0.31	0.03497
cea2.p.36256	F13H8.1		0.93	0.35	0.04305
cea2.i.10018	C44B7.4		0.93	0.25	0.02359
cea2.i.19217	E02H9.2		0.93	0.52	0.09064
cea2.p.149143	K03E6.7		0.93	0.15	0.00880
cea2.i.00251	C04F12.8		0.93	0.24	0.02226
cea2.d.09924	C01B4.7		0.92	0.11	0.00506
cea2.c.07603	Y53H1B.2		0.92	0.22	0.01906
cea2.p.110541	C37C3.6b	ppn-1	0.92	0.26	0.02473
cea2.d.04175	F58A4.7a	hlh-11	0.92	0.08	0.00279
cea2.d.10224	C02F5.7b		0.92	0.25	0.02336
cea2.d.28586	F55A12.9b	pqn-44	0.92	0.27	0.02869
cea2.p.82926	ZK688.2		0.91	0.36	0.04966
cea2.i.03836	K12C11.3		0.91	0.29	0.03174
cea2.p.131311	ZK287.2	sulp-8	0.91	0.31	0.03734
cea2.c.35448	F23B12.9	egl-1	0.91	0.42	0.06447
cea2.p.149794	K09C8.3	nas-10	0.91	0.39	0.05637
cea2.p.40619	F54C9.7		0.91	0.01	0.00006
cea2.p.52328	Y57A10B.1		0.90	0.25	0.02419
cea2.p.39987	F46C5.3	nas-25	0.90	0.18	0.01240
cea2.p.143962	F39D8.2		0.90	0.32	0.04043
cea2.d.16106	C39B10.6	elt-4	0.90	0.10	0.00382
cea2.d.05917	T23F2.2a		0.90	0.44	0.07217
cea2.p.52966	ZC101.2e	unc-52	0.90	0.35	0.04850
cea2.i.53180	F22F1.3		0.89	0.16	0.01032
cea2.p.139083	F02E8.6	ncr-1	0.89	0.33	0.04379
cea2.d.39707	T22H2.5b	scrm-1	0.89	0.11	0.00534
cea2.i.19113	C45G9.8		0.89	0.05	0.00102
cea2.3.08612	F08F1.1a	kin-9	0.89	0.17	0.01140
cea2.d.24229	F37A4.7a	rbf-1	0.89	0.39	0.05949
cea2.d.31706	K01A2.9		0.88	0.39	0.06012
cea2.i.31408	Y94H6A.2		0.88	0.36	0.05211
cea2.p.155993	W06B11.2	puf-9	0.88	0.07	0.00192
cea2.p.123405	T04C12.4	act-3	0.88	0.21	0.01771
cea2.i.12470	F44F4.1		0.88	0.25	0.02650
cea2.i.53884	F45E1.1		0.88	0.23	0.02294
cea2.d.32626	K07B1.6a		0.87	0.23	0.02347
cea2.d.08165	ZK622.3b	pmt-1	0.87	0.09	0.00333
cea2.p.56173	C05D10.3	wht-1	0.87	0.24	0.02525
cea2.d.05243	T04C9.4c	mlp-1	0.87	0.29	0.03470
cea2.p.95101	K02D7.1		0.87	0.37	0.05596
cea2.d.05232	T04A6.1a		0.86	0.24	0.02422
cea2.c.35298	F21C10.10		0.86	0.30	0.03824
cea2.d.07670	ZK867.1b	syd-9	0.86	0.27	0.03164
cea2.p.05416	C53D5.3		0.86	0.40	0.06578
cea2.i.17320	Y54E2A.9		0.86	0.41	0.06956
cea2.p.134311	C17H11.1		0.86	0.30	0.03901
cea2.p.75551	W09D6.5		0.85	0.08	0.00324
cea2.d.01969	F01G10.8	daf-14	0.85	0.10	0.00431

cea2.c.13730	R10H1.1		0.85	0.15	0.00974
cea2.i.54483	F53A9.6		0.85	0.10	0.00432
cea2.c.33504	C02A12.1	gst-33	0.85	0.28	0.03419
cea2.p.42654	K08F8.2	atf-2	0.85	0.13	0.00727
cea2.p.06426	F07A5.5	sue-1	0.84	0.17	0.01314
cea2.i.32787	C06H2.5	glb-3	0.84	0.19	0.01673
cea2.p.124225	T08B1.1		0.84	0.25	0.02787
cea2.p.71664	R107.8	lin-12	0.84	0.20	0.01917
cea2.3.00939	B0412.1		0.84	0.37	0.05793
cea2.p.149487	K07E3.8		0.84	0.20	0.01852
cea2.p.125924	T26F2.2		0.84	0.20	0.01894
cea2.p.39976	F46C5.2		0.83	0.15	0.01113
cea2.p.67921	K04G7.3		0.83	0.12	0.00670
cea2.p.115607	F31F7.2		0.83	0.20	0.01904
cea2.d.30162	F58A4.7b	hlh-11	0.83	0.14	0.00933
cea2.c.19448	F44B9.10		0.83	0.24	0.02618
cea2.c.32570	Y73B6BR.1		0.83	0.14	0.00967
cea2.d.14599	C27H5.2b		0.83	0.05	0.00147
cea2.p.28846	C01G12.1		0.82	0.25	0.02990
cea2.c.47068	M03F4.2a	act-4	0.82	0.02	0.00023
cea2.i.33384	C15C8.5		0.82	0.13	0.00846
cea2.p.135826	C34D10.1		0.82	0.23	0.02610
cea2.p.19665	W10D5.3a	gei-17	0.82	0.11	0.00569
cea2.p.43773	M176.4		0.82	0.19	0.01757
cea2.d.08041	T12A2.16b	hot-4	0.81	0.27	0.03433
cea2.i.51322	C34F6.6		0.81	0.25	0.02964
cea2.i.27358	K08D10.9		0.81	0.31	0.04588
cea2.i.52505	F09F9.3		0.81	0.17	0.01483
cea2.p.117933	F53F4.5	fmo-4	0.81	0.14	0.00945
cea2.d.06423	Y106G6A.2a		0.81	0.09	0.00452
cea2.i.00893	C46H11.3		0.81	0.03	0.00050
cea2.c.11310	F15A4.6		0.81	0.19	0.01860
cea2.c.47639	T08G2.3	acdH-10	0.81	0.19	0.01833
cea2.i.03274	F58H10.1		0.81	0.29	0.04139
cea2.p.137172	C43C3.2		0.80	0.31	0.04683
cea2.c.50234	C56C10.3	vps-32.1	0.80	0.21	0.02240
cea2.p.133460	C11E4.6		0.80	0.16	0.01248
cea2.d.48982	ZK354.5	msp-51	0.80	0.12	0.00743
cea2.c.03627	F43G9.9	cpn-1	0.80	0.14	0.01030
cea2.p.31758	C29H12.6		0.80	0.15	0.01101
cea2.p.117327	F46F3.1	ceH-27	0.80	0.20	0.02041
cea2.p.19369	W05F2.4		0.79	0.20	0.02095
cea2.c.47444	T02C5.1		0.79	0.05	0.00134
cea2.i.51711	C44H4.6		0.79	0.07	0.00244
cea2.3.15706	F53A10.2a		0.79	0.03	0.00053
cea2.d.34563	R05F9.3	msp-32	0.78	0.23	0.02661
cea2.p.122290	R09B5.11		0.78	0.17	0.01581
cea2.i.50487	C14F11.4		0.78	0.28	0.04146
cea2.d.32620	K07B1.6a		0.78	0.19	0.01949
cea2.c.27835	F28D1.7	rps-23	0.78	0.08	0.00361
cea2.i.38928	F41B5.10	nhr-183	0.78	0.02	0.00033
cea2.p.127431	Y17D7B.4		0.77	0.05	0.00144
cea2.p.42960	K10B4.3		0.77	0.17	0.01598
cea2.p.56766	C07H6.1	lig-4	0.77	0.17	0.01616
cea2.d.07939	F53A10.2b		0.77	0.15	0.01295
cea2.c.24738	Y75B8A.2b	nob-1	0.77	0.22	0.02579
cea2.d.38640	T12A2.16a	hot-4	0.77	0.04	0.00085
cea2.d.46624	Y71F9B.5b	lin-17	0.76	0.03	0.00043
cea2.p.09510	F32H2.9	tba-6	0.76	0.06	0.00221

cea2.p.107654	C04E6.7		0.76	0.08	0.00350
cea2.p.138147	C54D1.5	lam-2	0.76	0.14	0.01174
cea2.p.151397	R04E5.10		0.76	0.15	0.01272
cea2.d.32874	K08C9.7		0.76	0.26	0.03763
cea2.i.06463	Y53C10A.14		0.76	0.19	0.02127
cea2.p.145172	F46H6.1	rhi-1	0.76	0.04	0.00116
cea2.i.14640	R07G3.8		0.75	0.15	0.01283
cea2.c.26776	C35B1.6		0.75	0.11	0.00653
cea2.d.46962	Y73C8B.1		0.75	0.06	0.00182
cea2.p.71250	R13F6.5		0.75	0.08	0.00418
cea2.c.23338	T27E9.7	abcf-2	0.75	0.04	0.00098
cea2.c.13260	K02A2.5		0.74	0.10	0.00590
cea2.p.48612	W04H10.3		0.74	0.17	0.01660
cea2.p.95747	K09B3.2		0.74	0.07	0.00291
cea2.i.52996	F20B6.4		0.74	0.09	0.00450
cea2.3.06654	C48B4.4c	ced-7	0.74	0.11	0.00770
cea2.p.32184	C33C12.3		0.74	0.12	0.00841
cea2.c.46354	H03A11.1		0.74	0.06	0.00218
cea2.c.22331	R74.3		0.74	0.09	0.00455
cea2.i.09020	C13A10.2		0.73	0.04	0.00119
cea2.i.59949	M01B12.1		0.73	0.17	0.01677
cea2.c.11304	F14F11.2		0.73	0.09	0.00513
cea2.d.34091	M03F8.2a	pst-1	0.73	0.09	0.00536
cea2.c.24842	Y82E9BR.3		0.73	0.10	0.00658
cea2.i.06466	Y53C10A.2		0.72	0.12	0.00961
cea2.p.11930	F55C7.7d	unc-73	0.72	0.08	0.00362
cea2.p.154552	T21B6.1	dgn-1	0.72	0.03	0.00062
cea2.d.39355	T20D4.15		0.71	0.04	0.00090
cea2.p.152209	R160.7	lst-2	0.71	0.07	0.00336
cea2.i.01123	C50F2.8		0.70	0.11	0.00806
cea2.c.33662	C06B8.8	rpl-38	0.70	0.16	0.01638
cea2.c.27166	C53D6.2	unc-129	0.70	0.09	0.00526
cea2.p.109349	C18G1.2		0.70	0.11	0.00848
cea2.c.46624	K02G10.4a	flp-11	0.70	0.03	0.00055
cea2.c.13510	M28.6	lact-3	0.70	0.14	0.01268
cea2.p.90741	F19C7.8a		0.70	0.11	0.00774
cea2.p.03654	C35E7.4		0.69	0.08	0.00427
cea2.c.48513	Y23B4A.2		0.68	0.10	0.00708
cea2.i.19263	F02A9.1a		0.68	0.07	0.00344
cea2.d.44954	Y51H4A.24		0.68	0.07	0.00312
cea2.d.39395	T20G5.8		0.67	0.08	0.00452
cea2.c.47119	M163.3	his-24	0.67	0.04	0.00137
cea2.i.20264	M04D8.7		0.67	0.06	0.00316
cea2.c.26640	C33A12.2	nlp-35	0.66	0.08	0.00429
cea2.p.124574	T10H9.2	scd-2	0.66	0.06	0.00275
cea2.d.40726	T28F3.8		0.64	0.04	0.00148
cea2.d.29982	F58A3.1b	ldb-1	0.64	0.03	0.00099
cea2.c.23305	T27E9.1		0.64	0.03	0.00067

Table 3A-4

Genes downregulated with *nhr-25* RNAi

C. elegans (07/14/09)	Gene Ids	Gene Name	Average log2 change	Std Dev	P value
cea2.p.124717	T13F3.6		-6.12	0.92	0.00738
cea2.p.158050	C04E12.4		-5.54	1.16	0.01443
cea2.p.100105	W05E7.1	grd-3	-5.45	1.06	0.01228
cea2.p.132864	C05E11.4	amt-1	-5.45	0.34	0.00128
cea2.p.50848	Y48E1B.8		-5.34	1.19	0.01605
cea2.d.01807	C55B7.4a	acd-1	-5.20	0.76	0.00710
cea2.p.95102	K02D7.3	col-101	-5.12	0.92	0.01050
cea2.3.24008	T15D6.3	gly-17	-5.12	0.92	0.01050
cea2.i.18516	C02D5.3		-5.04	1.04	0.01398
cea2.d.30327	F59D8.1	vit-3	-4.98	1.15	0.01747
cea2.i.25170	F09D12.1	grd-10	-4.85	0.92	0.01186
cea2.3.17681	F59D8.2	vit-4	-4.73	1.12	0.01830
cea2.i.53381	F31B9.2		-4.71	0.91	0.01210
cea2.i.33883	C25F9.6		-4.67	0.85	0.01083
cea2.c.35135	F07C4.7	grsp-4	-4.59	0.50	0.00397
cea2.3.05195	C35A5.3		-4.51	0.62	0.00626
cea2.i.12394	F43C11.3		-4.41	1.37	0.03049
cea2.d.02954	F31F4.7	ugt-42	-4.34	0.71	0.00874
cea2.d.16119	C39D10.8c		-4.34	0.29	0.00151
cea2.i.32631	C04F5.7	ugt-63	-4.32	1.28	0.02819
cea2.p.149038	K02G10.7		-4.32	1.28	0.02819
cea2.i.08332	B0454.5		-4.31	1.53	0.03940
cea2.d.06866	Y50D4B.4		-4.18	0.77	0.01105
cea2.d.26120	F45D11.14		-4.15	0.60	0.00701
cea2.d.39373	T20D4.4		-4.08	1.91	0.06626
cea2.i.16393	W10G11.3		-4.01	1.24	0.03034
cea2.c.37003	K02E2.4	ins-35	-3.93	0.96	0.01944
cea2.p.75766	Y22D7AR.10		-3.90	0.73	0.01145
cea2.i.40997	K03B8.8		-3.88	2.56	0.11965
cea2.i.58976	T15B7.4	col-142	-3.88	0.86	0.01611
cea2.p.124718	T15B7.3	col-143	-3.87	1.07	0.02472
cea2.3.28999	Y50D4B.4		-3.84	0.00	0.00000
cea2.i.40252	F58E10.5	end-3	-3.83	0.86	0.01633
cea2.d.30360	F59D8.1	vit-3	-3.82	1.42	0.04302
cea2.d.00307	C05D10.1b		-3.73	1.02	0.02396
cea2.d.10485	C04E12.5		-3.71	0.85	0.01706
cea2.i.07622	ZK39.6	clec-97	-3.54	0.84	0.01847
cea2.p.124403	T09F5.9	clec-47	-3.52	0.70	0.01276
cea2.i.12232	F40H3.2		-3.51	0.62	0.01028
cea2.p.149938	K09F5.2	vit-1	-3.44	1.30	0.04467
cea2.i.40814	K01D12.14	cdr-5	-3.42	0.93	0.02349
cea2.i.53228	F23D12.1		-3.38	0.17	0.00082
cea2.i.59038	Y50D4B.3		-3.37	0.09	0.00023
cea2.d.07339	Y73B6BL.24		-3.35	0.69	0.01389
cea2.c.06702	W03F11.1		-3.27	1.60	0.07103
cea2.3.29007	Y50D4B.4		-3.22	0.76	0.01824
cea2.p.139499	F09E10.1		-3.18	0.00	0.00000
cea2.p.11540	F53G12.7	col-45	-3.11	0.19	0.00130
cea2.3.10341	F17C8.3		-3.10	1.32	0.05517
cea2.p.90769	F20B10.3		-3.09	0.54	0.00999
cea2.d.44373	Y47H9A.1		-3.07	0.24	0.00208

cea2.p.29997	C09G5.3	col-79	-3.07	0.24	0.00208
cea2.c.29167	F56B3.1	col-103	-3.03	0.71	0.01783
cea2.d.07549	ZK512.7		-3.02	0.42	0.00639
cea2.d.23702	F35F10.4		-3.00	1.15	0.04559
cea2.i.54590	F54B11.1	col-9	-2.98	1.31	0.05878
cea2.i.06621	Y53F4B.23		-2.97	0.99	0.03519
cea2.i.28894	W08E12.8		-2.95	1.06	0.04007
cea2.i.56646	T01B10.1	grd-4	-2.94	0.54	0.01112
cea2.d.08154	ZK1290.3a	rol-8	-2.88	0.16	0.00109
cea2.p.158067	C39E9.2	scl-5	-2.86	0.31	0.00401
cea2.i.30617	Y55F3AR.3	cct-8	-2.86	0.60	0.01419
cea2.p.154841	T22H6.7	abf-6	-2.84	0.96	0.03608
cea2.p.34911	F01D5.7		-2.84	0.10	0.00044
cea2.d.08178	F35D6.1b	fem-1	-2.83	0.61	0.01535
cea2.i.58837	F07C4.2	clec-45	-2.83	0.95	0.03558
cea2.d.39269	T19C9.4	srh-109	-2.80	1.19	0.05553
cea2.d.38519	T10B5.8		-2.78	1.73	0.10853
cea2.i.24189	C39E9.9	col-132	-2.78	0.20	0.00172
cea2.i.27375	K08D12.6		-2.77	0.15	0.00102
cea2.c.41465	C15H9.7		-2.77	0.50	0.01091
cea2.p.33109	C47D12.4		-2.76	0.91	0.03425
cea2.p.148451	H11E01.2		-2.71	0.51	0.01170
cea2.c.18330	F01F1.6	alh-9	-2.65	0.18	0.00156
cea2.i.34054	C31G12.2	clec-245	-2.64	1.59	0.10304
cea2.p.129931	Y69H2.14		-2.64	0.95	0.04090
cea2.i.24373	C44B12.3		-2.61	1.23	0.06656
cea2.d.31787	K02A2.1		-2.59	0.38	0.00718
cea2.i.57929	Y12A6A.1		-2.58	0.57	0.01611
cea2.i.59007	W08E12.9		-2.57	1.32	0.07755
cea2.3.17694	F59D8.2	vit-4	-2.56	0.94	0.04181
cea2.p.136035	C34F6.3	col-179	-2.55	0.69	0.02354
cea2.i.44491	T11F9.3	nas-20	-2.54	0.26	0.00340
cea2.p.154713	T22B7.7		-2.54	0.19	0.00178
cea2.p.100437	Y11D7A.3		-2.53	1.15	0.06202
cea2.p.30002	C09G5.5	col-80	-2.53	0.13	0.00082
cea2.i.18681	C09F5.3		-2.53	0.13	0.00082
cea2.i.58110	Y62H9A.7		-2.51	0.76	0.02923
cea2.i.55302	H28G03.3		-2.51	0.17	0.00147
cea2.c.43172	F09E10.6		-2.50	0.36	0.00685
cea2.p.116259	F37B4.7	fol-2	-2.50	1.34	0.08393
cea2.i.55662	K09A11.2	cyp-14A1	-2.50	0.49	0.01241
cea2.p.31798	C30G12.2		-2.48	0.62	0.02048
cea2.d.41954	Y105C5B.13	skr-10	-2.47	0.32	0.00553
cea2.i.00585	C27C7.3	nhr-74	-2.47	1.27	0.07808
cea2.i.48616	ZC404.7		-2.45	0.95	0.04653
cea2.i.36175	F10C2.7		-2.45	0.35	0.00677
cea2.i.32970	C10G8.7	ceh-33	-2.44	0.52	0.01484
cea2.c.16150	B0244.2	ida-1	-2.43	0.49	0.01314
cea2.d.49211	ZK488.10	pqn-97	-2.42	1.08	0.05997
cea2.p.78014	Y48G9A.1		-2.41	0.21	0.00251
cea2.p.69392	M88.4		-2.41	0.50	0.01383
cea2.c.32710	Y116A8C.32	sfa-1	-2.38	0.37	0.00793
cea2.p.158092	F43G6.7		-2.37	0.53	0.01655
cea2.i.47510	Y50E8A.1		-2.36	0.41	0.01009
cea2.d.22888	F32A5.2b		-2.35	0.19	0.00212
cea2.i.32961	C10G8.4		-2.35	1.43	0.10477
cea2.d.20991	F17A9.5		-2.35	1.25	0.08243
cea2.d.02156	F10C1.2a	ifb-1	-2.35	0.34	0.00688
cea2.i.55022	F58A3.5	ttr-31	-2.33	0.49	0.01446

cea2.p.129394	Y51A2D.18		-2.32	0.27	0.00462
cea2.p.139508	F09E10.3	dhs-25	-2.31	0.58	0.02032
cea2.p.36165	F11G11.11	col-20	-2.30	0.70	0.02944
cea2.i.35583	D1065.5	srh-211	-2.29	0.30	0.00573
cea2.i.27370	K08D12.6		-2.28	0.47	0.01401
cea2.c.23340	T28D6.3		-2.28	1.09	0.06882
cea2.p.129713	Y60A3A.10	dhs-24	-2.27	0.90	0.04881
cea2.i.29357	Y17G9A.3		-2.26	1.39	0.10589
cea2.d.00791	C23H3.2a		-2.26	0.38	0.00917
cea2.p.69922	R02F2.8		-2.25	0.34	0.00760
cea2.p.50376	Y48B6A.4	eat-2	-2.23	1.29	0.09590
cea2.i.38716	F40A3.2		-2.21	1.19	0.08411
cea2.d.29674	F57B1.3	col-159	-2.21	0.85	0.04656
cea2.p.138094	C54D1.4	alh-10	-2.20	0.49	0.01597
cea2.c.49373	ZK1193.1	col-19	-2.20	0.76	0.03729
cea2.p.128896	Y46H3B.1	clec-205	-2.20	0.56	0.02065
cea2.p.86637	C24F3.6	col-124	-2.19	0.87	0.04841
cea2.i.24912	C54E4.5		-2.17	0.23	0.00359
cea2.i.53541	F35C8.8		-2.16	0.28	0.00552
cea2.p.43822	M176.7	kin-16	-2.16	1.04	0.07009
cea2.i.13327	F58E1.4		-2.15	0.96	0.05997
cea2.d.24400	F38C2.1		-2.15	1.23	0.09439
cea2.d.30363	F59D8.1	vit-3	-2.15	0.72	0.03508
cea2.p.24760	Y105E8A.14		-2.15	0.55	0.02133
cea2.i.38062	F31F4.15	fbxa-72	-2.15	0.72	0.03544
cea2.c.41919	C34F6.2	col-178	-2.14	0.41	0.01204
cea2.c.37679	R08E5.3		-2.13	0.99	0.06494
cea2.i.58729	ZK899.1		-2.13	0.21	0.00320
cea2.p.88987	C50F7.4		-2.12	0.41	0.01250
cea2.p.134286	C17G1.6		-2.12	0.75	0.03917
cea2.d.46545	Y71D11A.3a		-2.11	0.39	0.01109
cea2.p.28647	C01B12.1	sqt-2	-2.11	0.19	0.00278
cea2.d.10603	C04F6.1	vit-5	-2.11	0.77	0.04151
cea2.c.18709	F14F7.1	col-98	-2.10	0.72	0.03722
cea2.i.24747	C50A2.3		-2.09	0.66	0.03194
cea2.d.07877	F25H5.1b	lim-9	-2.08	0.49	0.01796
cea2.p.89211	C53B4.5	col-119	-2.08	0.28	0.00594
cea2.p.87605	C35B1.4		-2.06	0.98	0.06750
cea2.d.07258	Y71F9B.13a		-2.05	0.35	0.00963
cea2.d.33301	K10D6.2c		-2.04	0.53	0.02178
cea2.i.38631	F38E1.6		-2.04	0.82	0.05035
cea2.i.15550	T16A1.4		-2.04	0.57	0.02475
cea2.d.07775	C23H5.8a		-2.04	0.27	0.00570
cea2.c.49414	T06E4.4	col-147	-2.03	1.15	0.09254
cea2.i.01479	E01A2.7		-2.03	0.23	0.00439
cea2.i.11520	F21D12.5		-2.03	0.54	0.02294
cea2.p.125267	T19H12.9	ugt-12	-2.03	0.20	0.00308
cea2.c.27549	F11E6.3		-2.01	0.43	0.01467
cea2.c.05035	K10D3.6		-2.00	0.49	0.01964
cea2.i.56514	R09F10.6	srh-11	-1.99	0.22	0.00409
cea2.i.21436	Y119D3B.16		-1.99	0.58	0.02697
cea2.c.07484	Y47H9C.4a	ced-1	-1.98	0.30	0.00735
cea2.i.55725	K09C4.5		-1.97	0.59	0.02858
cea2.p.40599	F54C9.4	col-38	-1.97	1.20	0.10445
cea2.d.07753	C10C5.5		-1.96	0.83	0.05459
cea2.d.05181	T01D1.2a	etr-1	-1.95	0.61	0.03091
cea2.d.06710	Y46H3B.2	clec-204	-1.95	1.03	0.08158
cea2.p.107877	C05E4.1	srp-2	-1.94	0.68	0.03809
cea2.i.59150	C13A2.11		-1.94	1.03	0.08241

cea2.d.44122	Y45G12C.12	str-154	-1.94	1.14	0.09883
cea2.i.02121	F32A7.7	str-245	-1.92	0.80	0.05268
cea2.i.59584	T21E8.2	pgp-7	-1.92	1.10	0.09492
cea2.p.17000	T10B11.1	pcbd-1	-1.91	0.42	0.01604
cea2.i.47322	Y47D7A.12		-1.91	0.64	0.03494
cea2.c.38575	T10H9.5		-1.91	0.03	0.00009
cea2.d.31437	H25K10.4		-1.91	0.66	0.03754
cea2.3.25579	W01F3.1a		-1.90	0.22	0.00462
cea2.p.102136	Y54G2A.6	clec-85	-1.90	0.35	0.01099
cea2.p.103373	Y67A10A.9		-1.90	1.02	0.08495
cea2.p.156984	ZC373.1		-1.90	1.01	0.08257
cea2.p.96418	R02D3.1		-1.89	0.75	0.04869
cea2.i.41779	K12B6.3	lips-4	-1.88	1.09	0.09643
cea2.p.81273	ZC21.3		-1.88	0.45	0.01865
cea2.p.131261	ZK228.3		-1.88	0.97	0.07824
cea2.d.16594	C43H8.3		-1.87	0.68	0.04105
cea2.i.26744	F58G6.2	srm-3	-1.86	0.66	0.03887
cea2.c.42461	C49F5.7		-1.86	1.09	0.09732
cea2.i.57727	T25G12.2		-1.86	1.09	0.09883
cea2.d.07741	C06A8.1b		-1.86	0.92	0.07351
cea2.p.152273	R193.2		-1.86	0.30	0.00853
cea2.p.128824	Y45G5AM.6		-1.85	0.69	0.04303
cea2.d.07303	Y71H10B.1a		-1.84	0.84	0.06290
cea2.d.21745	F22F7.1a		-1.84	0.78	0.05459
cea2.p.101902	Y45F10C.4		-1.82	0.99	0.08529
cea2.p.125242	T19H12.1		-1.82	0.11	0.00117
cea2.d.13978	C24B9.13		-1.82	0.46	0.02101
cea2.c.36595	F54D11.1	pmt-2	-1.82	0.58	0.03210
cea2.i.07060	Y71F9B.1		-1.82	0.89	0.07077
cea2.i.24881	C54E4.1		-1.82	0.90	0.07260
cea2.p.111924	D2023.7	col-158	-1.81	0.33	0.01096
cea2.p.10877	F47G4.2		-1.80	1.03	0.09400
cea2.c.40233	Y113G7A.4		-1.80	0.56	0.03118
cea2.c.22552	T03F6.1	qdpr-1	-1.79	0.48	0.02346
cea2.d.22879	F31F6.3		-1.79	0.87	0.07054
cea2.i.45823	W06G6.11		-1.78	1.09	0.10494
cea2.i.15234	T10B9.7	cyp-13A2	-1.77	0.63	0.03957
cea2.d.25300	F42F12.9	nspc-10	-1.77	0.26	0.00695
cea2.i.54251	F49H12.2		-1.75	0.77	0.05865
cea2.i.17993	ZK546.11	gst-30	-1.75	0.70	0.04928
cea2.p.47039	T14B4.5		-1.74	0.58	0.03462
cea2.p.150537	M03B6.2		-1.74	0.38	0.01563
cea2.p.105726	ZK185.3		-1.74	0.56	0.03338
cea2.d.05214	T02G5.9b	krs-1	-1.73	0.40	0.01766
cea2.p.57172	C14B9.6a	gei-8	-1.70	0.37	0.01503
cea2.p.115085	F26F12.1		-1.70	0.63	0.04247
cea2.i.46663	Y38C9B.1	cyp-29A3	-1.69	0.69	0.05189
cea2.p.42457	K06A1.3	cutl-16	-1.68	0.73	0.05723
cea2.d.37761	T07D4.4b	ddx-19	-1.67	0.51	0.02989
cea2.c.49390	C52D10.6	skr-12	-1.63	0.09	0.00111
cea2.i.52072	D1005.3		-1.63	0.27	0.00926
cea2.p.125142	T19B10.5		-1.62	0.72	0.05985
cea2.d.21817	F23H11.8a	pef-1	-1.62	0.11	0.00160
cea2.d.36544	T02G5.9a	krs-1	-1.61	0.50	0.03057
cea2.i.22631	Y82E9BR.19		-1.61	0.78	0.07067
cea2.i.44586	T15B7.5	col-141	-1.60	0.82	0.07718
cea2.i.42499	R08F11.4		-1.60	0.24	0.00772
cea2.i.00393	C10H11.3	ugt-25	-1.60	0.43	0.02298
cea2.i.59370	F40D4.6	srbc-25	-1.59	0.87	0.08689

cea2.i.41291	K07C11.1	pax-1	-1.59	0.41	0.02097
cea2.p.49633	Y38E10A.22		-1.58	0.49	0.03089
cea2.p.154562	T21B6.2		-1.58	0.20	0.00516
cea2.d.27012	F48E8.1c	lon-1	-1.58	0.62	0.04805
cea2.i.31210	Y73F8A.18		-1.57	0.64	0.05077
cea2.i.02306	F35C12.3		-1.57	0.28	0.01009
cea2.c.42305	C44H4.3	sym-1	-1.57	0.44	0.02514
cea2.p.36300	F13H8.3		-1.57	0.10	0.00137
cea2.p.86564	C24D10.1		-1.57	0.37	0.01814
cea2.i.35418	C55A6.4		-1.57	0.20	0.00539
cea2.p.94539	F58G6.7		-1.56	0.78	0.07449
cea2.i.30358	Y4C6B.3		-1.56	0.54	0.03750
cea2.i.18079	ZK669.2		-1.55	0.16	0.00374
cea2.p.140463	F14B8.4		-1.55	0.60	0.04734
cea2.d.03561	F47A4.1b	lgc-47	-1.54	0.45	0.02684
cea2.d.06340	W06F12.1a	lit-1	-1.54	0.42	0.02368
cea2.d.03988	F55A8.2d	egl-4	-1.54	0.48	0.03070
cea2.d.37261	T05H10.5a	ufd-2	-1.53	0.46	0.02904
cea2.c.18698	F11H8.3	col-8	-1.53	0.45	0.02703
cea2.d.12875	C16A3.10b		-1.53	0.36	0.01786
cea2.c.35530	F25B4.9	clec-1	-1.53	0.55	0.04080
cea2.p.91039	F23B2.11	pcp-3	-1.52	0.38	0.01991
cea2.3.30288	Y73B3A.2		-1.52	0.27	0.01018
cea2.i.25158	F08G5.5	ugt-65	-1.51	0.23	0.00732
cea2.d.26993	F48E8.1a	lon-1	-1.51	0.80	0.08198
cea2.i.05760	Y37D8A.3		-1.50	0.73	0.07012
cea2.p.72186	T04A8.5		-1.49	0.59	0.04853
cea2.d.27003	F48E8.1b	lon-1	-1.48	0.71	0.06866
cea2.c.46924	K10C2.4		-1.48	0.11	0.00178
cea2.p.81047	Y111B2A.14	pqn-80	-1.47	0.53	0.04033
cea2.3.18172	H19M22.2c	let-805	-1.47	0.45	0.02954
cea2.3.08449	F08A8.3		-1.46	0.12	0.00235
cea2.d.25296	F42F12.7	nspc-14	-1.46	0.36	0.01947
cea2.p.91046	F23B2.12	pcp-2	-1.46	0.83	0.09272
cea2.i.16454	Y110A2AM.1		-1.46	0.24	0.00903
cea2.d.37787	T07D4.4c	ddx-19	-1.46	0.71	0.07093
cea2.p.95377	K07H8.6		-1.46	0.68	0.06615
cea2.d.00692	C16A3.10a		-1.45	0.30	0.01353
cea2.p.123620	T05E12.7		-1.45	0.68	0.06594
cea2.3.18489	H38K22.5a	gly-6	-1.45	0.66	0.06212
cea2.d.46087	Y62E10A.13b		-1.45	0.42	0.02707
cea2.d.03982	F55A8.2c	egl-4	-1.45	0.08	0.00104
cea2.d.25290	F42F12.6	nspc-13	-1.45	0.33	0.01720
cea2.c.15186	Y38F1A.6		-1.44	0.26	0.01093
cea2.c.41121	C08A9.1	sod-3	-1.44	0.55	0.04603
cea2.i.16360	W10G11.16	lgc-45	-1.44	0.08	0.00098
cea2.p.158128	Y38C1AA.7		-1.43	0.24	0.00908
cea2.3.24526	T19D12.2		-1.43	0.45	0.03091
cea2.d.05735	T19A5.2a	gck-1	-1.43	0.45	0.03203
cea2.3.06753	C49D10.1		-1.43	0.27	0.01133
cea2.i.48550	ZC266.1		-1.40	0.19	0.00611
cea2.3.26256	W05B2.5	col-93	-1.40	1.08	0.15449
cea2.p.141282	F18E9.3		-1.40	0.34	0.01929
cea2.d.10161	C02E11.1b		-1.39	0.41	0.02768
cea2.p.119644	F59D6.3		-1.38	0.08	0.00109
cea2.d.02537	F19B6.1b		-1.38	0.48	0.03848
cea2.p.46040	T05H10.4		-1.38	0.57	0.05158
cea2.d.22876	F31F6.3		-1.38	0.40	0.02678
cea2.i.21898	Y48G9A.2		-1.38	0.19	0.00605

cea2.p.116685	F41F3.4	col-139	-1.38	0.47	0.03661
cea2.i.33047	C12D5.9		-1.37	0.12	0.00265
cea2.c.01371	C44E4.4		-1.37	0.11	0.00196
cea2.d.07840	F07A11.6d	din-1	-1.37	0.41	0.02788
cea2.i.52471	F09A5.3		-1.37	0.58	0.05445
cea2.3.22250	T01D1.2a	etr-1	-1.37	0.46	0.03563
cea2.p.28717	C01F1.3		-1.37	0.38	0.02524
cea2.3.24517	T19D12.2		-1.37	0.58	0.05580
cea2.d.04289	H19M22.2b	let-805	-1.36	0.10	0.00178
cea2.c.35152	F08F3.3	rhr-1	-1.36	0.45	0.03518
cea2.i.16974	Y48C3A.3		-1.36	0.38	0.02551
cea2.i.16474	Y17G7B.1	acbp-6	-1.35	0.59	0.05759
cea2.p.73101	T12A2.1		-1.35	0.09	0.00154
cea2.i.55790	K09E2.3		-1.35	0.34	0.02046
cea2.d.26093	F44F4.11	tba-4	-1.34	0.61	0.06178
cea2.d.07957	F57C9.1a		-1.34	0.17	0.00506
cea2.c.31653	Y24D9A.8a		-1.34	0.36	0.02316
cea2.d.00230	C01G5.2	prg-2	-1.34	0.58	0.05796
cea2.p.26264	ZK265.2	col-63	-1.33	0.54	0.05040
cea2.i.27470	K11H12.7		-1.33	0.20	0.00732
cea2.c.08121	Y71G12B.6		-1.33	0.47	0.03960
cea2.d.25933	F44E2.2c	retr-1	-1.33	0.28	0.01403
cea2.p.143104	F31F6.6	nac-1	-1.32	0.25	0.01146
cea2.d.00324	C05D11.1a		-1.32	0.45	0.03619
cea2.3.03626	C23H3.2a		-1.32	0.20	0.00752
cea2.d.40412	T27D12.2a	clh-1	-1.32	0.16	0.00496
cea2.p.25705	Y110A7A.19		-1.32	0.57	0.05656
cea2.p.28159	B0286.3		-1.31	0.15	0.00413
cea2.d.26502	F46F11.9a		-1.31	0.52	0.04793
cea2.c.36418	F46E10.2		-1.31	0.40	0.02993
cea2.p.97677	T05A1.2	col-122	-1.31	0.32	0.01962
cea2.c.49404	F57B1.4	col-160	-1.31	0.22	0.00932
cea2.d.47718	ZC404.3b	spe-39	-1.31	0.51	0.04701
cea2.i.23384	C07G1.7		-1.31	0.38	0.02751
cea2.3.23043	T07D4.4a	ddx-19	-1.31	0.54	0.05334
cea2.d.25287	F42F12.1	nspc-9	-1.30	0.29	0.01560
cea2.d.39239	T19B10.4b	pqn-70	-1.30	0.36	0.02473
cea2.i.31002	Y67D8C.7		-1.30	0.05	0.00059
cea2.i.57305	T19D7.1	lgc-32	-1.30	0.38	0.02729
cea2.p.135418	C30G4.4		-1.29	0.18	0.00619
cea2.p.76652	Y39A3CR.5		-1.29	0.09	0.00169
cea2.c.50416	C33H5.4		-1.29	0.31	0.01931
cea2.d.34165	M04C9.6b		-1.29	0.60	0.06601
cea2.p.143556	F38B6.4		-1.28	0.37	0.02595
SpotReport4LTP4			-1.28	0.54	0.05369
cea2.p.158120	W03D8.9		-1.28	0.58	0.06224
cea2.c.13453	K10H10.2		-1.27	0.54	0.05625
cea2.i.48639	ZC412.1		-1.27	0.13	0.00340
cea2.p.146879	F53B3.6		-1.27	0.25	0.01311
cea2.3.27519	Y24D9A.8a		-1.26	0.39	0.03026
cea2.d.39051	T17H7.4a	gei-16	-1.26	0.07	0.00106
cea2.p.140452	F14B8.3	pes-23	-1.26	0.31	0.01956
cea2.p.114346	F23B12.3		-1.26	0.19	0.00718
cea2.i.58702	ZK813.2		-1.26	0.41	0.03424
cea2.i.45697	W02H5.2		-1.26	0.35	0.02449
cea2.p.22492	Y54E5A.4	npp-4	-1.26	0.45	0.04044
cea2.p.124314	T08H10.1		-1.25	0.47	0.04319
cea2.3.08458	F08A8.3		-1.25	0.06	0.00077
cea2.p.85372	C06A6.4		-1.25	0.53	0.05457

cea2.d.34598	R06A4.4b	imb-2	-1.25	0.48	0.04535
cea2.d.34410	R01E6.3b	cah-4	-1.25	0.27	0.01564
cea2.3.03243	C17C3.1b		-1.24	0.39	0.03155
cea2.3.20553	M04F3.3		-1.24	0.57	0.06440
cea2.i.34633	C48G7.2		-1.23	0.67	0.08544
cea2.d.23895	F36D4.3a	hum-2	-1.23	0.40	0.03333
cea2.p.14410	K10C3.4		-1.23	0.67	0.08480
cea2.i.59232	C52E2.8		-1.23	0.26	0.01458
cea2.d.08095	Y37D8A.23a	unc-25	-1.23	0.13	0.00343
cea2.p.158213	F23A7.4		-1.23	0.24	0.01208
cea2.d.04847	R06A4.4a	imb-2	-1.23	0.43	0.03810
cea2.d.31866	K02F2.1b	dpf-3	-1.23	0.48	0.04690
cea2.3.09714	F14B8.3	pes-23	-1.22	0.16	0.00544
cea2.d.25932	F44E2.2c	retr-1	-1.22	0.34	0.02480
cea2.p.39129	F38A3.1	col-81	-1.22	0.33	0.02406
cea2.d.17237	C47C12.4		-1.22	0.45	0.04294
cea2.d.07967	H18N23.2b		-1.21	0.45	0.04383
cea2.d.16123	C39E9.14a	dli-1	-1.21	0.33	0.02329
cea2.p.85697	C08F11.11		-1.21	0.10	0.00241
cea2.i.40809	K01D12.13		-1.21	0.43	0.03949
cea2.d.45160	Y54E10A.9b	vbh-1	-1.20	0.42	0.03844
cea2.d.43813	Y43F4A.4		-1.20	0.28	0.01769
cea2.i.01535	F08A8.3		-1.20	0.35	0.02675
cea2.p.119166	F57B1.6		-1.20	0.38	0.03259
cea2.d.47255	Y87G2A.8		-1.19	0.37	0.03101
cea2.d.32222	K04G2.8a	apr-1	-1.19	0.57	0.06875
cea2.d.44971	Y53C12B.3a	nos-3	-1.19	0.42	0.03848
cea2.p.158245	H04D03.1		-1.19	0.19	0.00860
cea2.d.39616	T22B3.2a		-1.19	0.43	0.04137
cea2.i.41530	K09D9.2	cyp-35A3	-1.19	0.51	0.05642
cea2.d.05764	T19H12.10	ugt-11	-1.19	0.27	0.01633
cea2.d.23241	F33D4.2e	itr-1	-1.19	0.40	0.03588
cea2.i.05039	W01A8.6		-1.19	0.12	0.00341
cea2.p.139854	F11C7.2		-1.19	0.07	0.00102
cea2.p.99672	VT23B5.1		-1.18	0.48	0.05029
cea2.i.29842	Y38F2AR.8		-1.18	0.29	0.01973
cea2.3.08988	F11A1.3a	daf-12	-1.18	0.29	0.01938
cea2.d.43780	Y41D4B.19a	npp-8	-1.18	0.38	0.03349
cea2.p.92541	F42A6.3		-1.18	0.49	0.05328
cea2.d.12928	C16A3.1c		-1.18	0.38	0.03349
cea2.i.20257	M04D8.6	xbx-3	-1.18	0.23	0.01262
cea2.d.30368	F59E12.4a	npl-4.1	-1.17	0.37	0.03104
cea2.d.13057	C17B7.11	fbxa-65	-1.17	0.30	0.02141
cea2.p.86443	C17H12.8		-1.17	0.29	0.01998
cea2.d.00930	C26E6.9b	set-2	-1.17	0.35	0.02871
cea2.d.05752	T19E7.2a	skn-1	-1.17	0.46	0.04733
cea2.d.07190	Y67D8A.2a		-1.17	0.34	0.02704
cea2.p.45647	T04B8.3		-1.17	0.26	0.01608
cea2.p.108096	C06H2.4	fol-1	-1.17	0.26	0.01608
cea2.i.42192	R02F11.3		-1.17	0.24	0.01391
cea2.d.04625	K09G1.1b		-1.16	0.32	0.02412
cea2.i.27310	K07H8.5		-1.16	0.31	0.02325
cea2.d.04108	F57C12.5b	mrp-1	-1.16	0.38	0.03418
cea2.d.38048	T08D2.3		-1.16	0.51	0.05947
cea2.d.01552	C48E7.11		-1.15	0.18	0.00767
cea2.i.29820	Y38F2AR.12		-1.14	0.12	0.00385
cea2.d.04816	R05D3.11	met-2	-1.14	0.29	0.02161
cea2.c.48472	Y15E3A.4		-1.14	0.45	0.04922
cea2.3.06804	C49H3.5a	ntl-4	-1.14	0.45	0.04922

cea2.3.08729	F09F7.4b		-1.14	0.35	0.02985
cea2.i.57965	Y34B4A.1		-1.14	0.33	0.02741
cea2.p.34644	E01G4.4	pqn-27	-1.14	0.26	0.01697
cea2.3.03443	C18G1.4a	pgl-3	-1.13	0.30	0.02250
cea2.i.12811	F49E12.2	dod-23	-1.13	0.17	0.00762
cea2.d.25666	F44B9.1b	dpf-6	-1.13	0.17	0.00762
cea2.p.69751	R01H10.4		-1.12	0.13	0.00415
cea2.p.54193	ZK1248.13		-1.12	0.71	0.11167
cea2.d.08194	ZK1248.17		-1.12	0.11	0.00328
cea2.d.49707	ZK662.3a	nhr-48	-1.12	0.31	0.02478
cea2.p.41805	H12I13.4	fbf-1	-1.12	0.28	0.02025
cea2.d.07814	C45H4.14b		-1.12	0.18	0.00877
cea2.3.13875	F42A6.7b	hrp-1	-1.12	0.17	0.00777
cea2.d.04624	K09G1.1a		-1.12	0.47	0.05348
cea2.3.05017	C34C6.2		-1.12	0.16	0.00656
cea2.i.49986	C05D9.8		-1.12	0.15	0.00607
cea2.d.47250	Y87G2A.8		-1.11	0.39	0.03902
cea2.p.32325	C33F10.8		-1.11	0.39	0.03835
cea2.d.36725	T04A11.3		-1.11	0.31	0.02569
cea2.d.01788	C53D5.1a		-1.11	0.28	0.02016
SpotReport4LTP4			-1.11	0.27	0.01927
cea2.d.04036	F56A8.3a		-1.10	0.43	0.04823
cea2.d.08000	M01E11.7a	tag-163	-1.10	0.29	0.02244
cea2.i.52868	F18E9.2	nlp-7	-1.09	0.26	0.01865
cea2.p.32615	C38C6.2	atgp-2	-1.09	0.25	0.01635
cea2.i.51267	C33E10.2	fbxa-120	-1.09	0.25	0.01696
cea2.c.22651	T05G5.6	ech-6	-1.09	0.13	0.00474
cea2.c.34787	C52E4.2	mif-2	-1.09	0.08	0.00190
cea2.d.13337	C17G10.9d		-1.09	0.30	0.02445
cea2.d.07838	F01F1.10a	eng-1	-1.09	0.28	0.02203
cea2.d.39064	T17H7.4a	gei-16	-1.09	0.52	0.06895
cea2.p.127151	W07B8.1		-1.09	0.35	0.03264
cea2.3.09364	F12E12.7	sdz-11	-1.09	0.17	0.00844
cea2.d.24495	F39B2.4b	sur-2	-1.09	0.41	0.04410
cea2.p.26137	ZC434.8		-1.08	0.32	0.02809
cea2.c.49411	K10B3.8	gpd-2	-1.08	0.23	0.01482
cea2.p.146461	F52D2.4	gei-12	-1.08	0.31	0.02565
cea2.d.19909	F11C7.3a	vap-1	-1.07	0.09	0.00214
cea2.d.14679	C28H8.11b		-1.07	0.36	0.03571
cea2.d.33993	M03C11.4	tag-235	-1.06	0.23	0.01482
cea2.c.25497	ZK637.13	glb-1	-1.06	0.30	0.02589
cea2.i.54689	F54F7.2		-1.06	0.21	0.01275
cea2.p.135679	C33D12.2		-1.06	0.68	0.11374
cea2.p.118706	F54F3.3		-1.06	0.68	0.11374
cea2.p.28072	B0281.5		-1.05	0.23	0.01559
cea2.i.57295	T18D3.6		-1.05	0.23	0.01617
cea2.d.03421	F42G9.5a	alh-11	-1.05	0.31	0.02826
cea2.p.160175	K08D10.1		-1.05	0.37	0.03994
cea2.3.25595	W01F3.1a		-1.05	0.07	0.00169
cea2.d.05346	T06G6.11		-1.05	0.30	0.02630
cea2.d.47800	ZC477.9a	deb-1	-1.05	0.56	0.08303
cea2.d.03558	F46F6.1a	rme-4	-1.04	0.13	0.00542
cea2.d.05582	T13C5.5b		-1.04	0.32	0.03058
cea2.d.22965	F32B5.1		-1.04	0.24	0.01694
cea2.d.26544	F46F11.9b		-1.04	0.37	0.04043
cea2.3.26412	W06A7.3b	ret-1	-1.04	0.57	0.08711
cea2.d.31492	H28G03.1c		-1.04	0.29	0.02442
cea2.c.14625	VF13D12L.3		-1.04	0.35	0.03495
cea2.p.136087	C34F6.8		-1.04	0.19	0.01100

cea2.p.69490	M142.6	rle-1	-1.04	0.46	0.05955
cea2.c.13938	T02G5.8	kat-1	-1.04	0.35	0.03524
cea2.d.31596	H42K12.1b	pdk-1	-1.04	0.26	0.02013
cea2.d.10005	C01G6.6b	tag-165	-1.03	0.16	0.00771
cea2.c.49419	T25C8.2	act-5	-1.03	0.33	0.03302
cea2.c.49420	Y111B2A.2		-1.03	0.30	0.02644
cea2.i.29144	Y105C5B.6		-1.03	0.35	0.03594
cea2.3.22142	T01A4.1b	gcy-28	-1.03	0.20	0.01184
cea2.c.35158	F08H9.6	clec-57	-1.02	0.50	0.07093
cea2.3.17901	H12I13.4	fbf-1	-1.02	0.25	0.01920
cea2.i.17738	ZK1067.8		-1.02	0.23	0.01687
cea2.3.16440	F55A8.2c	egl-4	-1.02	0.30	0.02767
cea2.d.11403	C08A9.6		-1.02	0.20	0.01294
cea2.p.121007	K09H11.3	rga-3	-1.02	0.41	0.05074
cea2.3.17695	F59G1.1a	cgt-3	-1.02	0.47	0.06333
cea2.i.59476	K06B9.1	cyp-25A6	-1.02	0.18	0.01049
cea2.d.07357	Y73F8A.25a		-1.02	0.28	0.02360
cea2.3.29932	Y59E9AL.6		-1.02	0.27	0.02231
cea2.d.00771	C18G1.4a	pgl-3	-1.02	0.27	0.02271
cea2.p.07188	F16A11.3		-1.02	0.33	0.03265
cea2.d.39106	T17H7.4c	gei-16	-1.02	0.16	0.00774
cea2.d.39034	T17E9.2c	nmt-1	-1.01	0.38	0.04307
cea2.d.13466	C18A3.5b		-1.01	0.20	0.01327
cea2.3.04056	C25G4.10		-1.01	0.28	0.02400
cea2.i.57984	Y40C7B.4		-1.01	0.16	0.00871
cea2.p.58898	C34C12.3	pph-6	-1.01	0.39	0.04603
cea2.i.23274	C05G6.2		-1.00	0.37	0.04231
cea2.p.50601	Y48C3A.12		-1.00	0.05	0.00085
cea2.3.11784	F25H5.3b		-1.00	0.28	0.02449
cea2.p.21890	Y48G1C.8		-1.00	0.40	0.04859
cea2.d.06160	W01F3.1a		-0.99	0.49	0.07158
cea2.i.29911	Y40H7A.4		-0.99	0.37	0.04295
cea2.3.17969	H17B01.1a		-0.99	0.28	0.02616
cea2.d.04285	H17B01.1a		-0.99	0.27	0.02412
cea2.i.36934	F19B2.2		-0.99	0.18	0.01064
cea2.i.22819	ZK112.3		-0.99	0.27	0.02389
cea2.d.41472	W07B3.2d	gei-4	-0.99	0.17	0.00983
cea2.p.35974	F10G7.2	tsn-1	-0.99	0.33	0.03469
cea2.p.126981	W06D12.5	twk-33	-0.99	0.26	0.02206
cea2.d.24444	F39B2.4a	sur-2	-0.99	0.33	0.03491
cea2.3.31654	ZK546.2a		-0.99	0.32	0.03329
cea2.p.77953	Y48A6C.4		-0.98	0.43	0.05769
cea2.i.18573	C05H8.2		-0.98	0.22	0.01609
cea2.3.10639	F19B6.2b	ufd-1	-0.98	0.20	0.01356
cea2.i.25091	F02H6.4		-0.98	0.41	0.05323
cea2.p.22894	Y54E10A.15	cdt-1	-0.97	0.08	0.00253
cea2.p.77087	Y43F4B.3	set-25	-0.97	0.27	0.02489
cea2.d.18860	F07A11.6c	din-1	-0.97	0.34	0.03858
cea2.p.141776	F20D1.9		-0.96	0.30	0.03029
cea2.i.06329	Y48G1C.10		-0.96	0.42	0.05813
cea2.d.16630	C44C1.4b	vps-45	-0.96	0.07	0.00178
cea2.3.31193	ZK1127.7	cin-4	-0.96	0.21	0.01548
cea2.p.116548	F40G12.11		-0.96	0.06	0.00125
cea2.i.53352	F31A3.3		-0.96	0.20	0.01455
cea2.d.18767	F07A11.6b	din-1	-0.96	0.35	0.04177
cea2.d.04358	H39E23.1b	par-1	-0.96	0.16	0.00871
cea2.p.128121	Y39B6A.39		-0.96	0.32	0.03470
cea2.c.18217	C56G2.6	let-767	-0.96	0.11	0.00455
cea2.d.01138	C33A12.6	ugt-21	-0.96	0.10	0.00392

cea2.p.103326	Y67A10A.4	fbxa-83	-0.95	0.21	0.01563
cea2.p.151863	R09F10.4	inx-5	-0.95	0.46	0.06899
cea2.d.04386	H43E16.1		-0.95	0.49	0.07881
cea2.p.158205	F20B4.7		-0.95	0.14	0.00757
cea2.c.32682	Y105C5B.28		-0.95	0.23	0.01990
cea2.d.03950	F54E7.3b	par-3	-0.94	0.33	0.03892
cea2.3.07221	C54D2.5b		-0.94	0.43	0.06458
cea2.p.20457	Y37E3.10		-0.94	0.24	0.02110
cea2.d.07954	F56D12.5b	vig-1	-0.94	0.11	0.00482
cea2.c.50266	W10C8.5		-0.93	0.03	0.00039
cea2.d.08325	AC8.1		-0.93	0.35	0.04361
cea2.d.03711	F49H12.6b	acl-4	-0.93	0.16	0.01022
cea2.p.130538	Y113G7B.21		-0.93	0.25	0.02313
cea2.i.44862	T20B3.6		-0.93	0.25	0.02313
cea2.3.14498	F45E4.11		-0.93	0.14	0.00785
cea2.d.45432	Y54G2A.17b		-0.93	0.38	0.05031
cea2.p.79920	Y71H2B.1		-0.93	0.11	0.00465
cea2.i.30643	Y55F3BL.3		-0.93	0.22	0.01820
cea2.p.07042	F14B4.3		-0.93	0.36	0.04652
cea2.p.102537	Y55F3AR.2		-0.93	0.08	0.00235
cea2.d.02173	F11C1.5a		-0.93	0.20	0.01564
cea2.d.27477	F52C9.8b	pqe-1	-0.92	0.26	0.02576
cea2.d.45008	Y53F4B.30	gst-27	-0.92	0.16	0.01000
cea2.3.13974	F42G9.5a	alh-11	-0.92	0.00	0.00000
cea2.d.13476	C18A3.5c		-0.91	0.18	0.01264
cea2.c.21904	M88.1	ugt-62	-0.91	0.18	0.01216
cea2.d.10046	C01G8.5a	erm-1	-0.91	0.25	0.02492
cea2.d.01786	C53B4.4c		-0.91	0.26	0.02511
cea2.p.114026	F20G2.2		-0.91	0.15	0.00945
cea2.p.05135	C48B6.3		-0.91	0.33	0.03997
cea2.d.07935	F52E4.1a	pccb-1	-0.91	0.23	0.02040
cea2.p.158203	F20B4.2		-0.91	0.30	0.03543
cea2.d.39093	T17H7.4b	gei-16	-0.91	0.10	0.00438
cea2.p.157279	ZC506.3	pssy-1	-0.91	0.09	0.00295
cea2.3.24530	T19E7.2a	skn-1	-0.91	0.34	0.04446
cea2.3.22886	T05F1.6a		-0.91	0.34	0.04333
cea2.p.00938	B0511.14		-0.90	0.25	0.02378
cea2.d.39075	T17H7.4b	gei-16	-0.90	0.12	0.00606
cea2.d.25858	F44E2.2b	retr-1	-0.90	0.21	0.01846
cea2.p.70422	R10E4.11		-0.90	0.06	0.00127
cea2.d.30748	H02I12.8	cyp-31A2	-0.90	0.19	0.01508
cea2.d.24236	F37B1.2	gst-12	-0.90	0.18	0.01305
cea2.i.59089	ZK945.8		-0.90	0.17	0.01230
cea2.3.31505	ZK520.4a	cul-2	-0.90	0.17	0.01159
cea2.3.08970	F11A1.3a	daf-12	-0.89	0.24	0.02400
cea2.d.15719	C36B1.8b		-0.89	0.31	0.03882
cea2.i.17718	ZC239.5		-0.89	0.31	0.03830
cea2.d.42564	Y17G9B.3	cyp-31A3	-0.89	0.30	0.03613
cea2.p.43753	M151.1		-0.89	0.03	0.00030
cea2.c.41795	C24H10.5	uvt-2	-0.89	0.20	0.01654
cea2.3.11661	F25D7.3	blmp-1	-0.88	0.21	0.01767
cea2.3.30470	Y73F8A.25a		-0.88	0.28	0.03297
cea2.i.49427	6R55.2		-0.88	0.28	0.03144
cea2.d.12452	C12D12.2a	glt-1	-0.88	0.17	0.01267
cea2.p.41234	F56D12.1		-0.88	0.17	0.01275
cea2.p.69261	M03C11.3		-0.88	0.26	0.02733
cea2.d.40496	T27F7.2a	shc-2	-0.87	0.11	0.00542
cea2.d.08024	R148.5a		-0.87	0.19	0.01480
cea2.d.02538	F19B6.2a	ufd-1	-0.87	0.16	0.01119

cea2.i.57893	W06D11.3		-0.87	0.25	0.02635
cea2.p.111888	D2023.1		-0.87	0.13	0.00775
cea2.c.03944	F53F10.3		-0.87	0.12	0.00656
cea2.d.44573	Y48G1BL.1		-0.87	0.37	0.05465
cea2.d.43005	Y23H5A.8b		-0.86	0.21	0.01925
cea2.p.29439	C06C3.5		-0.86	0.07	0.00210
cea2.d.42554	Y17G7B.2c	ash-2	-0.86	0.20	0.01826
cea2.d.08054	T21C9.3a		-0.86	0.05	0.00123
cea2.d.14193	C25A8.1		-0.86	0.18	0.01468
cea2.d.06705	Y45G5AM.8		-0.86	0.23	0.02290
cea2.i.11726	F29C12.4		-0.86	0.21	0.02038
cea2.p.126294	W01A11.5	cpt-6	-0.85	0.20	0.01694
cea2.c.00939	C30F8.2	vha-16	-0.85	0.26	0.02979
cea2.i.04174	T02E1.4		-0.85	0.32	0.04432
cea2.i.49080	ZK287.3		-0.85	0.16	0.01109
cea2.c.48220	T24D8.5	nlp-2	-0.85	0.16	0.01109
cea2.d.33155	K09H9.1		-0.85	0.04	0.00074
cea2.i.66612	Y73B6BL.2	htp-2	-0.84	0.27	0.03354
cea2.i.06295	Y48G1BM.6		-0.84	0.17	0.01261
cea2.d.31678	JC8.6a	lin-54	-0.84	0.25	0.02902
cea2.p.24411	Y92H12A.5		-0.84	0.15	0.00987
cea2.p.137236	C44C1.1		-0.84	0.14	0.00884
cea2.c.32312	Y66H1B.4	spl-1	-0.84	0.11	0.00619
cea2.d.01170	C34C6.2		-0.84	0.30	0.03998
cea2.i.55475	K04C1.5		-0.84	0.07	0.00225
cea2.p.137009	C41G11.1		-0.84	0.07	0.00215
cea2.d.46741	Y71H2AM.20b		-0.83	0.17	0.01360
cea2.c.47321	R11.1		-0.83	0.13	0.00809
cea2.d.46837	Y73B3A.4		-0.83	0.28	0.03687
cea2.d.11556	C09B8.7a	pak-1	-0.83	0.18	0.01517
cea2.p.158278	K08D8.5		-0.82	0.14	0.00966
cea2.d.05774	T20B3.2	tni-3	-0.82	0.13	0.00861
cea2.p.119234	F57F5.1		-0.82	0.13	0.00808
cea2.p.99154	T22D1.11		-0.82	0.13	0.00808
cea2.c.49410	K10B3.7	gpd-3	-0.82	0.11	0.00569
cea2.i.19741	F53A2.5	dro-1	-0.82	0.08	0.00340
cea2.p.18995	W03F11.5b		-0.82	0.04	0.00076
cea2.i.02104	F32A7.1		-0.82	0.18	0.01641
cea2.c.06048	T20F10.2		-0.82	0.15	0.01143
cea2.i.31013	Y69A2AR.10		-0.81	0.11	0.00652
cea2.p.06753	F11C3.2	unc-122	-0.80	0.08	0.00327
cea2.i.34933	C50H11.15	cyp-33C9	-0.80	0.20	0.02024
cea2.p.114974	F25H9.2		-0.80	0.28	0.03719
cea2.d.13183	C17E7.9b		-0.80	0.19	0.01814
cea2.p.60369	C56G2.15		-0.80	0.18	0.01566
cea2.d.29898	F57C9.1b		-0.80	0.23	0.02608
cea2.c.32647	Y77E11A.15	col-106	-0.80	0.21	0.02151
cea2.p.39159	F40F8.4		-0.80	0.20	0.02104
cea2.d.02523	F18H3.5a	cdk-4	-0.79	0.19	0.01881
cea2.d.38088	T08D2.7		-0.79	0.15	0.01213
cea2.3.25530	VT23B5.1		-0.79	0.11	0.00675
cea2.p.58927	C34C12.8		-0.79	0.09	0.00411
cea2.d.06742	Y47G6A.22b		-0.78	0.15	0.01190
cea2.c.04962	K07A3.1	fbp-1	-0.78	0.10	0.00554
cea2.p.155754	W03G11.1		-0.78	0.10	0.00554
cea2.d.00958	C28C12.9a	acdh-13	-0.78	0.04	0.00078
cea2.d.14695	C29A12.3b	lig-1	-0.77	0.15	0.01173
cea2.3.16387	F55A8.2a	egl-4	-0.77	0.13	0.00909
cea2.i.02998	F55D12.1		-0.77	0.08	0.00359

cea2.d.37902	T08A9.6		-0.77	0.06	0.00217
cea2.p.73621	T16H12.2		-0.77	0.18	0.01808
cea2.p.117916	F53F1.2		-0.77	0.13	0.00958
cea2.d.40786	W02A11.4b		-0.76	0.10	0.00571
cea2.c.23856	Y39E4B.3		-0.75	0.05	0.00149
cea2.i.65970	Y47H9C.14		-0.75	0.05	0.00149
cea2.d.20486	F13G3.11		-0.74	0.13	0.01016
cea2.c.22725	T07C4.7	mev-1	-0.74	0.07	0.00317
cea2.i.04076	R06A10.1		-0.74	0.05	0.00142
cea2.3.06163	C44E4.7		-0.73	0.09	0.00557
cea2.d.39839	T23E7.2b		-0.72	0.08	0.00382
cea2.d.46113	Y62E10A.14		-0.71	0.07	0.00370
cea2.d.22972	F32B5.1		-0.71	0.05	0.00163
cea2.d.46926	Y73B6BL.5b	seu-1	-0.70	0.05	0.00160
cea2.d.00789	C23G10.2a		-0.70	0.05	0.00160
cea2.d.36120	R166.5b	mnk-1	-0.70	0.04	0.00088
cea2.i.05958	Y43F11A.3		-0.69	0.10	0.00738
cea2.c.28543	F36H12.1	nlp-47	-0.68	0.11	0.00840
cea2.i.48558	ZC266.2		-0.68	0.05	0.00168
cea2.i.30878	Y62E10A.17		-0.68	0.04	0.00089
cea2.c.14417	T24H7.1	phb-2	-0.68	0.08	0.00409
cea2.d.05734	T18H9.7a	tag-232	-0.67	0.02	0.00039
cea2.p.55687	C02C2.3	cup-4	-0.66	0.05	0.00174
cea2.d.48273	ZK1128.2a		-0.65	0.05	0.00219

Chapter IV: PI3 Kinase Regulation of Intestinal Lipid Storage

Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of conserved lipid and protein kinases that preferentially phosphorylate phosphoinositol in the 3' position. This family of proteins plays a central role in many cellular signaling pathways, regulating processes including growth, autophagy, metabolism, vesicular trafficking and ageing. PI3Ks are capable of phosphorylating phosphoinositol, PI(4)P and PI(4,5)P to generate PI(3)P, PI(3,4)P and PI(3,4,5)P, with different classes of PI3Ks exhibiting differing substrate preference, and potentially having differential access to each potential substrate (Engelman et al., 2006). A number of protein domains have been found to bind 3' phosphorylated phosphoinositides, including FYVE finger, pleckstrin homology, and PX domains (Engelman et al., 2006; Rameh and Cantley, 1999; Roggo et al., 2002). Phosphorylation of phosphoinositides by PI3Ks leads to recruitment of proteins containing these domains to specific membranes, facilitating signaling cascades.

The PI3K family is comprised of 3 classes (I, II and III) as well as a number of protein kinases that are related by sequence to the PI3Ks, but do not phosphorylate lipids. Class III is comprised of a single catalytic member, Vps34, which is found in all eukaryotes, and is thought to be ancestral to all PI3Ks (Engelman et al., 2006). Vps34 was originally identified in yeast, as a component of the endocytic pathway (Backer, 2008). Vps34 has subsequently been shown to regulate other processes, including the TOR pathway and autophagy (Backer, 2008). In *C. elegans*, Vps34 (also named *let-512*) has been confirmed to function both in the endocytic pathway and in autophagy (Hansen et al., 2008; Roggo et al., 2002).

The class I PI3Ks are likely the most studied members of the PI3K family. This class has a single member in *C. elegans*, *age-1*, four catalytic members in mammals, P110 α , β , δ , and γ , and is not present in yeast. This class functions downstream of either receptor tyrosine kinases or G-protein coupled receptors. The *C. elegans* genome encodes a single Class I PI3K, AGE-1, which functions downstream of the Insulin/IGF receptor homolog DAF-2, and upstream of PI3 dependant kinase PDK-1, which acts on AKT-1, AKT-2 and SGK-1, ultimately regulating the FOXO transcription factor DAF-16. This pathway is a central regulator of dauer development and ageing, as well as lipid storage (Hu, 2007; Kenyon, 2010).

Class II PI3Ks are the least understood members of the family. Both *C. elegans* and mammals have a single member, F39B1.1 and Pi3KC2 respectively. The mammalian class II PI3K has been shown to bind to clatherin, and localize to coated pits, suggesting a role in receptor internalization (Engelman et al., 2006). The *C. elegans* class II member has not been studied, and no phenotypes are ascribed to the available knockout allele.

In this study, we have utilized the PI3K inhibitor LY294002, a broad spectrum PI3K inhibitor (Knight et al., 2006). We began examining the effects of this drug in an effort to manipulate the insulin-signaling pathway to study its role in metabolism. However, we found that this drug causes a significant reduction in Nile Red staining, which cannot be suppressed by loss of *daf-16*, indicating that its effects are not mediated solely through the canonical *C. elegans* insulin/IGF pathway. Our findings demonstrate that the Nile Red reducing effect of LY294002 is dependent on the putative sugar transporter K09C4.5. We show that this protein functions cell autonomously in the

intestine, and localizes to the Nile Red staining gut granules. We also find that K09C4.5 is necessary for the Nile Red reduction caused by RNAi mediated knockdown of the small gtpase *rab-7*. These findings clearly demonstrate that K09C4.5 regulates phenotypes associated with gut granules. The underlying mechanisms accounting for these phenotypes remain to be elucidated.

Results

LY294002 treatment leads to reduced Nile Red staining

In order to examine the role of PI3 kinase signaling on metabolic processes, we tested the effect of the commercially available PI3 kinase inhibitor LY294002 on Nile Red staining. We found that treatment with this compound caused a significant reduction in Nile Red staining in wild type animals (figure 4-1). This effect became noticeable at concentrations as low as 5 μ M (data not shown) while at 25 μ M LY294002 treatment caused a more robust reduction in staining, and 50 and 100 μ M caused dramatic reductions in staining (data not shown, and figure 4-1).

The most thoroughly characterized PI3 kinase in *C. elegans* is the sole class I family member *age-1*. Because AGE-1 is a key component of insulin/IGF signaling, a pathway regulating numerous physiological processes including metabolism, we wished to test whether the observed reduction of Nile Red staining upon LY294002 treatment could be attributed to inhibition of this protein. However, we found that the hypomorphic *age-1(hx546)* allele did not exhibit significantly reduced Nile Red staining (data not shown). The FOXO transcription factor *daf-16* functions downstream of *age-1* to mediate all the effects of insulin/IGF signaling on ageing and development (Lin et al.,

1997; Ogg et al., 1997). If the reduced Nile Red staining caused by treatment with LY294002 was the result of inhibition of AGE-1, loss of DAF-16 would be expected to suppress the effects of the compound. However, when we treated *daf-16 (mgDf47)* mutants with LY294002, we observed a reduction in Nile Red staining equivalent to that seen in wild type animals (data not shown). Combined, these data indicate that the Nile Red reduction caused by LY294002 is not the result of inhibition of AGE-1 alone.

***ft6* mutants are insensitive to the Nile Red reducing effect of LY294002**

Though LY294002 likely inhibits some or perhaps all members of the PI3 kinase family to varying degrees, the mechanism by which this leads to reduced Nile Red staining was unclear. In order to identify components of a pathway required for this effect, we conducted a forward mutagenesis screen. We assayed the F2 progeny of mutagenized wild type animals treated with 25 μ M LY294002 on Nile Red plates. We found a recessive mutant, *ft6*, which exhibited a relatively wild type level and pattern of Nile Red staining, but was insensitive to the Nile Red reduction caused by LY294002 treatment (figure 4-2).

***ft6* has a splice site mutation in K09C4.5**

Using SNP mapping, we localized the *ft6* mutation to between X: -11.59 and X: -10.50 (figure 4-3). This region is completely spanned by 6 cosmids, one of which, K09C4 appeared to rescue *ft6* mutants. We knocked down each of the 11 genes contained in cosmid K09C4 using RNAi in wild type animals, and only knockdown of K09C4.5 appeared to phenocopy *ft6* mutants. Sequencing of K09C4.5 revealed a G $\rightarrow$ A mutation in 3' splice site of the 3rd predicted intron.

To test the effect of the splice site mutation, we cloned 4 cDNAs from *ft6* mutants and 4 from wild type animals. Sequencing of these cDNAs revealed that exon 3, 3' to the splice site mutation is missing in *ft6* cDNAs. In addition, 2 of the 4 sequenced *ft6* cDNAs also were missing the last 54 bases of the upstream exon 3 (figure 4-3). All of the misspliced *ft6* cDNAs are frameshifted near the 5' end almost certainly creating a null allele.

K09C4.5 is similar in sequence with the mammalian GLUT family and microbial inositol tranporters

K09C4.5 is an uncharacterized gene. Based on its sequence it is predicted to function as a sugar transporter. In a blast against the human proteome, the top 5 hits are all members of GLUT family of facilitated sugar transporters.

Because K09C4.5 resembles sugar transporters, and the *C. elegans* genome encodes more than 20 genes with considerable sequence similarity to sugar transporters, we considered the possibility that inhibition of other sugar transport might suppress LY294002 induced Nile Red reduction. To test this, we generated an RNAi sublibrary of these putative transporters, and tested the effect of knockdown of each gene in LY294002 treated wild type animals stained with Nile Red (table 4-1). We found that only knockdown of K09C4.5 produced any suppression of the LY294002 Nile Red reduction (data not shown). Though we did not verify knockdown of each of these genes, these data suggest K09C4.5 plays a unique role among predicted sugar transporters in mediating the effect of LY294002.

The human GLUT family, also known as the SLC2A family, is comprised of 13 members. A majority of these proteins have been characterized, some more thoroughly

than others. GLUT4 is the most thoroughly studied mammalian sugar transporter. It is the primary insulin-responsive glucose transporter. Upon insulin stimulation, GLUT4 translocates from its basal-state intracellular location to the cell membrane, leading to insulin stimulated glucose uptake (Huang and Czech, 2007). GLUT4 exhibits high affinity glucose selective transport (Huang and Czech, 2007; Wood and Trayhurn, 2003). GLUT1 is a ubiquitously expressed high affinity glucose transporter, while GLUT2 exhibits a more restricted expression pattern and is a lower affinity glucose transporter (Wood and Trayhurn, 2003). GLUT5 is the only member of the family to show strong affinity for fructose and very low affinity for glucose (Wood and Trayhurn, 2003). GLUT8, formerly named GLUTX1, is a more recently characterized member of the GLUT family. Its localization appears to be exclusively intracellular, and translocation to the cell membrane has not been observed upon insulin stimulation, raising the possibility that its function is transport of sugar across intracellular membranes (Ibberson et al., 2000). The biological process or processes that require intracellular sugar transport are unclear, yet the existence of transporters apparently localized exclusively intracellularly suggests an unappreciated complexity in sugar compartmentalization within cells. GLUT8 has been demonstrated to transport glucose in a *X. leavis* oocyte system, but not other common sugars (Ibberson et al., 2000). The only member of the GLUT family that has been demonstrated to transport a solute other than glucose or fructose is HMIT, the proton-coupled *myo*-inositol transporter (Uldry et al., 2001). HMIT is unique among GLUT family members, both because it transports *myo*-inositol, and because its transport activity is coupled to proton transport, making it the only GLUT member that utilizes an ion gradient to transport its cargo.

An unbiased blast search against the NCBI protein database reveals that K09C4.5 exhibits stronger homology to a number of microbial and plant transporters than it does to the human GLUT family members. Two of these transporters have been characterized biochemically. The *Bacillus subtilis* transporter *iolT* is a *myo*-inositol transporter. K09C4.5 is also highly homologous to the *Arabidopsis thaliana* gene *AtVGT1*, which has been demonstrated to transport glucose and to be localized intracellularly on the vacuolar membrane. Sequence similarity alone however is insufficient to confidently predict which sugar K09C4.5 transports, and until transport activity is demonstrated the possibility that this protein transports an unrelated small molecule remains.

K09C4.5 is expressed in the intestine and expression of the wild type gene rescues *ft6*

We wished to determine the tissue(s) in which K09C4.5 is expressed. To examine expression, we fused a 2kb region of the upstream regulatory sequence to GFP, and generated transgenic animals containing this construct. These animals exhibited GFP expression only in the posterior of the intestine, an expression pattern that may be associated with the *unc-54* 3'UTR included in most *C. elegans* expression constructs. Based on the expression of this construct, we suspected that K09C4.5 might indeed be expressed in the intestine, but perhaps either at low levels, or in a manner that requires additional regulatory elements, potentially from within introns of the K09C4.5 gDNA.

To further explore the expression pattern of K09C4.5, as well as to confirm that loss of K09C4.5 is in fact responsible for the LY294002 insensitivity in *ft6* animals, we wished to rescue the mutant with a wild type copy of the gene. We generated a construct containing a 4.6kb K09C4.5 promoter region driving the K09C4.5 gDNA fused to GFP (plasmid pKA264). Injections of this construct proved uniformly lethal. In order to

obtain lines carrying this construct, two approaches were employed. Microparticle bombardment can be used to generate transgenic animals carrying low copy integrated constructs (Praitis et al., 2001). Using the bombardment method, we generated only a single transgenic line, but unfortunately the construct was not integrated. We also performed injections after which the P0 injected animals were placed on K09C4.5 RNAi plates. F1 animals carrying the coinjection marker were singled onto plates without RNAi, and from these we obtained two independent transgenic lines, KQ526 and KQ527 that exhibited faint intestinal GFP expression in F3 generation. Presumably GFP expression became evident as a result of reduced RNAi mediated knockdown of the construct two generations following removal from the RNAi plates. Though initial observations indicated KQ526 and KQ527 exhibited equivalent phenotypes, only KQ526 was used for initial characterization of K09C4.5. Following maintenance of this strain for a period of a few months, a spontaneous integration event occurred in KQ526, and subsequent characterization was conducted using the integrated strain KQ545.

To test the ability of this construct to rescue *ft6* mutants, we placed transgenic animals on plates containing LY294002 and Nile Red. While *ft6* mutants are normally resistant to the Nile Red reducing effect of LY294002, we found that transgenic KQ526 animals were responsive, exhibiting significantly reduced Nile Red staining in response to LY294002 treatment (figure 4-4). We also examined Nile Red staining in transgenic animals not treated with drug. While KQ526 Nile Red staining was not dramatically different from that of non-transgenic animals, a modest reduction in Nile Red staining was evident in transgenic animals. This reduction was largely restricted to those intestinal cells expressing the transgene at the highest levels. In most animals, the

anterior pair or two pairs of intestinal cells expressed K09C4.5::GFP strongest and showed reduced Nile Red staining (figure 4-4).

K09C4.5 functions cell autonomously within intestinal cells

In the earliest generations, before transgene expression had stabilized, we observed a number of mosaic animals. When treated with LY294002 and stained with Nile Red, these animals clearly revealed that cells expressing the transgene were fully rescued (i.e. they stained very little with Nile Red), while non-transgenic cells exhibited the full *ft6* mutant phenotype (insensitivity to LY294002 and bright Nile Red staining) as shown in figure 4-5. These mosaic animals demonstrate that K09C4.5 functions cell autonomously.

K09C4.5 localized to intracellular vesicles that stain with Nile Red

In transgenic animals expressing, K09C4.5::GFP exhibited primarily intracellular localization. Rarely was GFP localization on the cell membrane observed, and in these few animals, only very faint GFP was detectable on the cell membrane. Within intestinal cells, K09C4.5::GFP localized to large vesicle structures, as well as to many smaller puncta (figure 4-6). Both the large vesicles and the smaller puncta appeared to be distributed evenly throughout intestinal cells, with no apparent bias for either apical or basal localization.

The integrated transgenic line KQ545 exhibited fairly consistent expression across animals and generations. However, within an animal, the anterior and posterior intestine typically exhibited stronger expression than did the medial intestine. In adult animals, the strongly expressing intestinal cells rarely contained very many if any large GFP positive vesicles, but rather contained many small bright puncta. In cells of the

medial intestine with lower expression, large GFP positive vesicles were much more abundant.

GFP expression was only observed in intestine throughout development and in adult animals. In early embryos, no GFP expression is apparent, until approximately the late comma stage. By pretzel stage, fairly strong GFP expression is evident, and this GFP is localized to large vesicles, with very few smaller GFP positive puncta present. In Larval stages L1 and L2, GFP localization continues to be primarily in large vesicles, but by L3 and L4 stages small GFP positive puncta become more prevalent.

When stained with Nile Red, the large intestinal vesicles to which K09C4.5::GFP localized to clearly are stained with Nile Red (figure 4-6). Nearly all, if not all Nile Red staining structures are K09C4.5 positive, yet many of the small K09C4.5::GFP labeled puncta fail to stain with Nile Red. In general, cells expressing K09C4.5::GFP at a high level, lack large GFP positive vesicles, and also lack Nile Red staining. Cells with more moderate K09C4.5::GFP have some large GFP positive vesicles, but these generally stain dimly with Nile Red. Cells with the lowest K09C4.5::GFP expression have the brightest Nile Red staining.

K09C4.5 does not colocalize with LMP-1, exhibits very little colocalization with RAB-5 and moderate colocalization with RAB-7

We suspected that K09C4.5::GFP labeled organelles may be part of the endocytic pathway. To examine this we crossed KQ545 animals with RT1103, a strain with an integrated intestinally driven *RFP::rab-7* construct, and RT1239, a strain with an integrated intestinally driven *RFP::rab-5* construct (a gift of Barth Grant). Both these constructs exhibit a significant degree of diffuse cytoplasmic RFP, but also localization to

endocytic compartments. RAB-5 localizes to early endosomes, while RAB-7 localizes to late endosomes. Neither construct showed strong colocalization with K09C4.5::GFP. RFP::RAB-5 colocalized in a very limited manner, with perhaps ~5-10% of GFP and RFP overlapping. RFP::RAB-7 colocalized to a larger extent with K09C4.5::GFP, with perhaps ~25-50% overlap (figure 4-6). These results clearly demonstrate that K09C4.5 localization is not limited to endosomal compartments, but suggests that the protein may be trafficked through these organelles.

Lysosome membrane associated protein homolog 1 (LMP-1) is localized to a subset of *C. elegans* lysosomes. To test whether K09C4.5 localizes to these structures, we generated an intestinally driven *lmp-1cDNA::mCherry* construct. This was injected into strain KQ545 to generate a strain expressing both K09C4.5::GFP and LMP-1::mCherry. However, analyses of these animals demonstrated no significant overlap between K09C4.5 and LMP-1 (figure 4-7).

The best-characterized protein that localizes to Nile Red containing gut granules is PGP-2 (Schroeder et al., 2007). *pgp-2* encodes an ABC transporter similar to the mammalian multi drug resistance gene MDR1. Hermann and colleagues identified mutations in this gene in a screen for animals that lack autofluorescent gut granules (Schroeder et al., 2007). They demonstrated that the PGP-2 protein localizes to the autofluorescent gut granules, which are also the organelles that stain with Nile Red. In *pgp-2* mutants, very few Nile Red staining gut granules remain. For this reason, it has been proposed that PGP-2 is required for biogenesis of gut granules (Schroeder Hermann 2007).

PGP-2 has been shown to be localized to the membrane of the Nile Red staining gut granules (Schroeder et al., 2007). In our hands, we find overexpression of the wild type *pgp-2::GFP* to disrupt gut granules, resulting in granules of abnormal shape and size, with very little Nile Red staining. We received from Greg Hermann a *pgp-2* cDNA construct with two mutations (K440R and K1069R) that should disrupt the transport activity of PGP-2. We generated transgenic animals carrying this construct, and found that it localized to Nile Red staining gut granules without disrupting either staining or morphology of these granules. Based on the localization of this construct to Nile Red gut granules, we feel it is likely that PGP-2 and K09C4.5 will colocalize on these structures. To test this directly, we have generated a *pVha-6::mCherry::pgp-2(K440R, K1069R)* construct which would allow us to directly test mCherry::PGP-2 and K09C4.5::GFP colocalization. However, we have not generated this strain.

Both K09C4.5 and PGP-2 localize to Nile Red staining gut granules. Loss of *pgp-2* leads to loss of Nile Red staining, while loss of *K09C4.5* promotes wild type Nile Red staining under conditions that normally have very low staining (i.e. LY294002 treatment, and *rab-7* RNAi as shown below). For these reasons we wanted to determine whether loss of K09C4.5 could suppress the low Nile Red staining of *pgp-2* mutants. To do so, we crossed *ft6* mutants with *pgp-2(gk114)* to obtain strain KQ1887. When stained with Nile Red, KQ1887 was indistinguishable from *pgp-2(gk114)* mutants, demonstrating that *pgp-2* is epistatic to K09C4.5 (figure 4-8). If *pgp-2* is indeed required for gut granule biogenesis, loss of K09C4.5 clearly is insufficient to bypass this requirement.

If PGP-2 is required for gut granule biogenesis, it is possible that in *pgp-2* mutants, K09C4.5 may be unable to localize appropriately. To test this, we crossed

KQ545 animals with *pgp-2(gk114)* to generate *pgp-2(gk114)* mutants carrying the K09C4.5::GFP construct (strain KQ1884). We found that K09C4.5::GFP was somewhat disrupted by mutation of *pgp-2* (figure 4-9). *pgp-2* mutant embryos exhibited a more severe disruption in K09C4.5. In otherwise wild type animals, K09C4.5 localizes almost exclusively to gut granules in embryos, however in *pgp-2* mutant embryos, very little intracellular K09C4.5::GFP is apparent, and a significant amount of GFP fluorescence is visible on the cell membrane of embryonic intestinal cells (figure 4-9). In adults in an otherwise wild type background, gut granules are distributed throughout intestinal cells. In a *pgp-2(gk114)* background K09C4.5::GFP positive vesicles were distributed unevenly, with some areas completely lacking these structures, while they clumped in other areas (figure 4-9). It would be interesting to examine the identity of these K09C4.5::GFP positive granules. They may constitute bone fide gut granules that fail to stain with Nile Red because they lack PGP-2. Alternatively, they could be an endosomal structure through which K09C4.5 is normally trafficked, and into which it is backed up in a *pgp-2* mutant background. Use of additional markers of endosomal structures could help to better define these structures.

An RNAi screen identifies *rab-7* as a gene functioning in the K09C4.5 pathway

Our findings demonstrate that K09C4.5 is required for the Nile Red reduction caused by treatment with LY294002. This drug has been demonstrated to inhibit PI3 kinases in mammalian cells, so we expect that K09C4.5 functions downstream of one or more members of this kinase family. We wished to identify additional components of this pathway. To do so, we performed two RNAi screens.

For the first screen, we tested each of the RNAi clones that increase Nile Red staining, as identified in Ashrafi et al. (Ashrafi et al., 2003). *ft6* has a mutation in K09C4.5, which was also identified as an RNAi clone that increases Nile Red staining. For this reason, we hoped that this subset of RNAi clones would be enriched for LY294002 suppressors. However, of these 112 clones, only inactivation of K09C4.5 inhibited the reduced Nile Red phenotype caused by LY294002.

For the second screen, we tested whether the *ft6* mutation could suppress the Nile Red reduction for each of the 305 RNAi clones identified by Ashrafi et al. as fat reducers (Ashrafi et al., 2003). We hoped that *ft6* would be insensitive to the Nile Red reduction caused by some of these RNAi constructs, just as it is insensitive to the Nile Red reduction caused by LY294002 treatment. Of these 305 clones, *ft6* suppressed the Nile Red reduction caused by knockdown of *rab-7* (figure 4-10).

Knockdown of *rab-7* leads to a significant reduction in Nile Red staining. RAB-7 is a necessary component in the trafficking of proteins through the endocytic pathway (Chavrier et al., 1990). RAB-7 may have a role in the maturation of early endosomes to late endosomes/multivesicular bodies. Loss of RAB-7 in mammalian cells has been demonstrated reduce recycling of the EGF receptor, leading to accumulation of the protein in late endosomal compartments coupled with reduced lysosomal degradation (Vanlandingham and Ceresa, 2009).

Initial observations indicate that RNAi mediated knockdown of *rab-7* leads to increased levels of GFP in KQ545 transgenic animals (data not shown). Though this would need to be repeated and quantified, this observation suggests that RAB-7 may function in the degradation of K09C4.5 in a similar manner as has been demonstrated for

the EGF receptor (Vanlandingham and Ceresa, 2009). An increased level of K09C4.5 protein could lead to reduced Nile Red staining. Furthermore, if RAB-7 functions in the trafficking of K09C4.5 from late endosomal compartments, and if these are the sites at which K09C4.5 functions to regulate Nile Red staining, knockdown of *rab-7* may also be altering the localization of K09C4.5 in a manner that increases its function.

LY294002 inhibits FYVE::GFP localization

As mentioned above, RAB-7 is an important protein in the regulation of the endocytic pathway. Of note is the fact that RAB-7 forms a functional complex with VSP-34, a class III PI3 kinase and its adaptor protein p150 (Stein et al., 2005; Stein et al., 2003). VPS-34 phosphorylates phosphoinositide (PI) at the 3 position to generate PI(3)P. This step is required for maturation of endosomes in the endocytic pathway. This relationship also raises the possibility that LY294002 inhibition of VPS-34 could lead to inhibition of RAB-7 function. Mammalian VPS-34 is inhibited by LY294002, as are all mammalian PI3 kinases to various degrees (Knight et al., 2006).

To test whether LY294002 inhibits *C. elegans* VPS-34 *in vivo*, we made use of a transgenic line carrying a 2X FYVE::GFP reporter construct driven by the heat shock inducible promoter *hsp16-2* (line FR480). FYVE domains bind PI(3)P, and therefore this fusion protein localizes to endocytic compartments containing this lipid. In wild type animals, following heat shock, GFP is localized to numerous rings, most clearly visible in the hypodermis. However, when animals are treated with LY294002 GFP localization is clearly reduced and disrupted (figure 4-11). This demonstrates that LY294002 likely inhibits VPS-34 *in vivo*, and supports a model by which this drug inhibits the function of RAB-7, which in turn regulates degradation and/or localization of K09C4.5.

LY294002 treatment induces expression of K09C4.5

In the process of testing K09C4.5::GFP expressing transgenic line KQ526, we observed that LY294002 treated animals exhibit significantly brighter intestinal GFP fluorescence. To examine this, we grew synchronized KQ545 animals to the young adult stage, and transferred them to plates containing either DMSO control, or LY294002 at concentrations of 25, 50 and 100 μ M. No obvious change in GFP intensity was observed at 2 hours, but by 4 hours, animals treated with either 50 or 100 μ M were clearly brighter than the DMSO treated control animals. Images from animals treated with 100 μ M for 5 hours are shown in figure 4-12.

Increased GFP levels could indicate either an increased expression of K09C4.5, reduced degradation of the protein (or possibly both). To examine the effect of LY294002 treatment on expression of K09C4.5, we grew wild type animals on 50 μ M LY from the L1 stage to L4, and measured expression of K09C4.5 using RT-PCR. We found that LY294002 treated animals exhibited a 4-fold increase in K09C4.5 expression (figure 4-12). We repeated this experiment using a range of LY294002 concentrations (5, 25 and 100 μ M). K09C4.5 induction varied with concentration, but surprisingly the highest dose of drug (100 μ M) produced the smallest increase in expression (1.5 fold) while the lowest dose (5 μ M) produced the largest increase in expression (12 fold). 25 μ M LY294002 increased expression 6.3 fold (data not shown). These data suggest there may be a negative feedback mechanism that dampens expression of K09C4.5 over time. Perhaps a short exposure of a few hours would produce different results. The mechanism by which feedback occurs may be worth investigation.

Genetic inhibition of PI3 kinase signaling induces K09C4.5 expression

Though LY294002 is a PI3 kinase inhibitor, its effects could be the result of inhibition of PI3 kinases, protein kinases in the PI3 kinase family such as *atm-1* or perhaps off target effects outside the PI3 kinase family. If upregulation of K09C4.5 by drug treatment is the result of inhibition of PI3 kinase signaling, genetic manipulations that reduce PI3 kinase signaling should likewise affect K09C4.5 expression level. A recent paper that examined expression profiles of *daf-2* (as well as other) mutants reported that *daf-2* exhibit a 2-fold increased level of K09C4.5. These data however were not confirmed with RT-PCR or any other method. DAF-2, the insulin/IGF receptor activates the class I PI3 kinase AGE-1, so loss of DAF-2 would lead to reduced PI3 kinase signaling through this pathway. In order to confirm that K09C4.5 is indeed upregulated in *daf-2* mutants, we compared expression in wild type (N2), *daf-16(mgDf47)*, *daf-2(e1370)* and *daf-2(e1370); daf-16(mgDf47)*. Animals were grown at 15 degrees for 48 hours to allow *daf-2(e1370)* to bypass dauer, then upshifted to 25 degrees overnight in order to reduce DAF-2 activity. We found that *daf-2(e1370)* animals exhibited 3.4-fold higher expression of K09C4.5 than wild type animals. Further, the induction of K09C4.5 in *daf-2(e1370)* was dependent on *daf-16* (figure 4-13). These results demonstrate that genetic inhibition of PI3 kinase signaling is sufficient to induce upregulation of K09C4.5 expression.

Though the increased expression of K09C4.5 caused by loss of *daf-2* is dependent on *daf-16*, the LY294002-induced reduction in Nile Red staining is not dependent on *daf-16*. LY294002 may increase expression of K09C4.5 through inhibition of multiple PI3 kinases, or it may alter K09C4.5 activity via an additional mechanism, such as increasing its stability or altering its localization.

ft6* partially suppresses the hazy Nile Red staining of *daf-2(e1370)

daf-2(e1370) animals exhibit reduced Nile Red staining, and a “hazy” staining pattern. Because we found that loss of *daf-2* activity induces expression of K09C4.5, and because increased K09C4.5 function leads to reduced Nile Red staining, we wished to determine whether the dim, hazy Nile Red staining of *daf-2(e1370)* was the result of increased K09C4.5 expression. To test this, we knocked down K09C4.5 expression using RNAi in *daf-2(e1370)* animals. This RNAi appeared to weakly suppress the *daf-2(e1370)* phenotype, leading to slightly brighter and more punctate staining (perhaps ~10-20% suppression). These data demonstrate that *daf-2(e1370)* animals require K09C4.5 activity to exhibit the full Nile Red phenotype, but that a majority of this phenotype is K09C4.5 independent.

LY294002 reduces BODIPY-fatty acid staining and autofluorescence in a K09C4.5 dependent manner

LY294002 greatly reduces Nile Red staining in wild type animals, but this effect is suppressed by loss of K09C4.5. What exactly this reduced Nile Red staining indicates is unclear. To further characterize the change in intestinal gut granules upon LY294002 treatment, we tested the effect of the drug using other fluorescent stains of this compartment.

BODIPY-fatty acids stain Nile Red staining gut granules in the intestine, as well as other intestinal lipid depots and lipid droplets in the hypodermis (personal observations and personal communication from George Lemeiux). We stained LY294002 and DMSO vehicle treated wild type and *ft6* animals with BODIPY-fatty acids. We found that LY294002 did in fact reduce BODIPY-fatty acid staining of wild type animals, but this

effect was fairly strongly suppressed in *ft6* mutant animals (figure 4-14). (LY294002 treatment causes animals to be a bit smaller and slightly delayed, which affects comparisons between DMSO treated and LY294002 treated animals.)

C. elegans intestinal cells exhibit significant autofluorescence, emitting across a wide spectrum but brightest in the blue range. The majority of this fluorescence arises from Nile Red staining gut granules. We examined the effect of LY294002 treatment on autofluorescence intensity, and found that drug treatment reduces autofluorescence in a manner dependent on K09C4.5 (figure 4-14).

To test the effect of drug on lysotracker staining, we grew wild type and *ft6* mutant animals on either 100 μ M LY294002 or DMSO vehicle with 2 μ M Lysotracker Red (Invitrogen). Following 48 hours of growth, to the L4 stage, animals were picked onto plates without Lysotracker and allowed to clear their intestinal lumen for 2 hours before imaging. Surprisingly, treatment with LY294002 strongly increased lysotracker staining in both wild type and *ft6* mutant animals, while vehicle treated wild type and *ft6* mutants were indistinguishable (figure 4-14). This indicates that LY294002 is having K09C4.5 independent effects on the endocytic pathway, in addition to K09C4.5 dependant affects on gut granules.

Combined, the findings that LY294002 reduces Nile Red staining, BODIPY-fatty acid staining and autofluorescence strongly indicate that this drug is affecting gut granules. These structures are labeled by both stains, and are the primary autofluorescent structures within the animal. In addition, the localization of K09C4.5::GFP to this structure further suggests that LY294002 and K09C4.5 are mediators of gut granule biology in some manner.

LY294002 does not alter TAG levels in a manner detectable by TLC

The reduction in Nile Red staining caused by LY294002 treatment could be the result of a number of processes. Nile Red is a lipid stain, and though at the concentrations used in these experiments it stains only gut granules, the change in staining could be indicative of an equally significant change in overall lipid storage levels. To examine this, we performed TLC with wild type and *ft6* mutant animals, treated with either 50 μ M LY294002 or DMSO vehicle. 50 μ M rather than 100 μ M was used to reduce the effect of the drug on growth and development. Nonetheless, drug treated animals were slightly delayed, and allowed to grow for 2 hours longer than vehicle control animals to compensate for this effect. Though members of the Ashrafi Lab have improved the TLC method significantly, this method of TAG determination remains fairly variable. To overcome this variability, 9 replicates of each condition were included in the assay. It should also be noted that even with improved methodology and a large number of replicates, this assay is likely able to detect only changes greater than 10-15%. These caveats aside, we were able to detect no significant difference in TAG levels between wild type and *ft6* mutant animals, nor between LY294002 and DMSO vehicle treated conditions (figure 4-15). These results rule out the possibility that a large change in total TAG levels underlies the change in Nile Red staining observed with LY294002 treatment. It remains possible, or even likely, that local changes in lipid storage and distribution within gut granules, which may not be detectable with TLC, underlie the changes in Nile Red staining.

We also investigated the possibility that LY294002 could be altering fatty acid oxidation rates. However, when we compared wild type animals grown either on DMSO vehicle, or 50 μ M LY294002, we found no significant difference (figure 4-15).

K09C4.5 ectopically expressed in *Saccharomyces cerevisiae* localizes to the vacuole, and fails to permit growth in a hexose uptake deficient strain

Though K09C4.5 is a predicted sugar transporter, whether or not it does transport a sugar, and if so which sugar it transports are unknown. To attempt to investigate this, we expressed the K09C4.5 cDNA in yeast deficient in hexose transport. This yeast strain cannot grow with common sugars, including glucose, fructose and galactose as the sole carbon sources (Wieczorke et al., 1999). Expression of human glucose transporters GLUT2 and GLUT4 in this yeast line rescues its ability to grow on glucose (Wieczorke et al., 2003). We hoped that by expressing K09C4.5 in this yeast line we would be able to determine whether or not it transports any of these common sugars. To do so, we generated a yeast expression construct with K09C4.5::mCherry driven by the adh promoter (pKA555). However, when transformed into the sugar uptake deficient yeast, this construct failed to rescue growth on glucose, fructose or galactose. Microscopy of this transgenic yeast strain revealed that the K09C4.5::mCherry fusion protein was strongly localized to the yeast vacuolar membrane. Failure to rescue growth may result from the intracellular localization of this transporter.

The human GLUT2 and GLUT4 wild type transporters were also unable to rescue growth of this strain on glucose. It was only upon introduction of mutations that forced these transporters to the cell membrane that this yeast strain could begin to grow with a glucose carbon source (Wieczorke et al., 2003). Dileucine motifs drive internalization of

a number of mammalian GLUTs, including GLUT4, GLUT6 and GLUT8 (Huang and Czech, 2007; Ibberson et al., 2000; Joost and Thorens, 2001; Wood and Trayhurn, 2003), and are also components of yeast internalization signals (Reggiori et al., 2000). The K09C4.5 protein sequence contains 3 dileucine motifs within the first 30 amino acids of the N-terminus. We mutated these dileucines to dialanines singly, in combinations of two, as well as making a construct in which all three dileucines are mutated to dialanine (plasmids pKA1148, pKA1149, pKA1158, pKA1159 and pKA1160). However, when yeast were transformed with these constructs, none of them exhibited apparent cell membrane localization of the K09C4.5::mCherry fusion protein (data not shown). Mutation of all three dileucines did cause yeast to grow very slowly, with colonies appearing only after 5 days at 30 degrees following transformation, suggesting this manipulation did in fact alter the function of K09C4.5. Nonetheless, microscopy revealed that K09C4.5::mCherry was primarily localized to small intracellular puncta in yeast expressing this construct. In addition, transformation of hexose uptake deficient yeast with each of these constructs failed to rescue growth on glucose, fructose and galactose (data not shown). It is possible that these dileucines are part of the internalization signal, but apparently additional mutations are necessary to force cell membrane localization.

LY294002 treatment alters expression of a small number of metabolic enzymes in a K09C4.5 independent manner

If LY294002 treatment is altering metabolic function, it may be doing so by altering expression of metabolic enzymes. If so, these changes may be suppressed by loss of K09C4.5. To test this we isolated RNA from wild type and *ft6* mutants, both

untreated, and treated with 100 μ M of LY294002. We examined expression of ~100 metabolic enzymes using RT-PCR (Van Gilst et al., 2005a; Van Gilst et al., 2005b). We found that LY294002 treatment induced changes in expression of 8 of these genes, but for the most part these changes were not dependent on K09C4.5, as similar changes were observed in *ft6* mutants. In addition, comparison of wild type untreated animals with *ft6* untreated mutants revealed no differences in expression. Of note is the fact that the largest expression change observed in LY294002 treated animals is an ~10 fold increase in T08B1.6 (*acs-3*) expression. Perhaps this provides a clue as to the relationship between this drug and *acs-3* mutants discussed in chapter 3. In sum, these RT-PCR data demonstrate that LY294002 does in some way alter metabolic pathways, but whether these changes are relevant to the K09C4.5 pathway is unclear.

A second mutagenesis screen identifies additional lines resistant to LY294002 induced Nile Red reduction

Though K09C4.5 is clearly required for the Nile Red reduction caused by LY294002 treatment, the mechanism through which it acts remains unclear. The changes in Nile Red may in fact not indicate a primary shift in lipid metabolism, as TLC analysis reveals no dramatic change in TAG levels in drug treated animals. In addition, the localization of K09C4.5 to Nile Red staining gut granules indicates that this protein may impact the function or identity of this organelle. In order to better understand how K09C4.5 functions, we sought to identify additional genetic components acting downstream of LY294002, and along with K09C4.5. To do so, we conducted a second EMS mutagenesis screen, and again screened for animals that exhibited relatively wild type Nile Red staining even when treated with LY294002. To improve selection from

the first screen, we increased the dose of LY294002 to 50 μ M, a dose that dramatically reduces Nile Red staining. From this screen we identified more than 20 new lines exhibiting suppression of the LY294002 induced reduction in staining (table 4-2). Identification of the causative mutations in these lines should shed additional light on the mechanism by which K09C4.5 functions to regulate Nile Red staining.

Discussion

The role of metabolism is to provide energy for cellular and organismal processes, such as growth and development, reproduction and movement. To efficiently operate such a system, an organism must store excess energy when possible to provide for periods of nutrient deprivation. In addition, during periods of low nutrient availability, energy usage must be shifted to allow survival, generally at the expense of other processes, such as growth, development and reproduction.

The most basic units of the metabolic orchestra are enzymes that convert nutrients such as fatty acids and sugars into ATP. While these steps are ultimately critical for an organism's metabolic success, numerous other processes are required for regulation and completion of this task. A molecule of sugar, a fatty acid or an amino must be first taken up across the intestinal lumen; decisions must then be made regarding the fate of that nutrient. It has to be targeted to the appropriate machinery for immediate consumption, storage, utilization in synthesis of cellular structural components or transport to another cell or tissue. It is therefore not surprising, given the complex tasks metabolic pathways must accomplish, that an enormous network proteins, configured into a densely interconnected framework, with built in redundancy and feedback loops and impacting

nearly all aspects of cell biology, is required to carry out the metabolic processes of an organism.

The tools we have available to tackle this network are relatively simple, and at times inadequate. Most assays of metabolic status, such as biochemical determination of TAG or phospholipid content, measurements of oxygen consumption, rates of fatty acid oxidation or oxygen consumption, measures of *de novo* synthesis of fatty acid and rates of fatty acid uptake, lack the resolution necessary to monitor these processes in organelles, single cells, single tissues or even single animals. Utilization of various lipid stains coupled with microscopy can allow for improved resolution, permitting subcellular resolution at times, but such approaches are only capable of addressing certain questions, and must be interpreted carefully. Multiple cellular processes can impact dye staining patterns and intensities, and definitively determining the cellular changes that underlie a change in staining can be challenging. Despite these limitations, in this study we have begun to elucidate a pathway regulating some aspect of metabolism in *C. elegans*.

In this project we show that treatment of animals with the broad spectrum PI3 kinase inhibitor LY294002 greatly reduces Nile Red lipid staining in intestinal gut granules. We performed a forward mutagenesis screen, and identified a mutant line, *ft6*, that is insensitive to the Nile Red reducing effect of drug treatment (figure 4-2). Using positional cloning, we found that *ft6* has a mutation in K09C4.5, an uncharacterized gene encoding a putative sugar transporter. The *ft6* mutation is in a predicted splice site, and we verified that this mutation leads to missplicing near the 5' end of the K09C4.5 cDNA, almost certainly making *ft6* a null allele (figure 4-3).

Expression of the wild type cDNA in *ft6* mutants revealed a number of things. We found that expression of this gene rescued the *ft6* mutants, confirming mutation of K09C4.5 is in fact causing resistance to LY294002 treatment. Transgenic animals clearly exhibited GFP expression limited to the intestine, demonstrating that this is the tissue in which K09C4.5 functions (figure 4-4). We also found a number of mosaic animals expressing the K09C4.5::GFP transgene in a subset of intestinal cells, and in these animals, transgenic cells were clearly rescued, while non-transgenic cells exhibited the mutant phenotype (figure 4-5). This demonstrates that K09C4.5 acts cell autonomously in intestinal cells. In addition, by comparing transgenic animals treated with LY294002 with those treated with DMSO vehicle, we found that LY294002 leads to increased K09C4.5::GFP fluorescence (figure 4-12). We performed RT-PCR to measure expression of the transgene in drug treated animals, and found that LY294002 induced expression of K09C4.5 (figure 4-12). These data demonstrate that drug treatment does induce expression of K09C4.5, but it remains possible (and we suspect it is likely the case) that LY294002 also interferes with turnover of the K09C4.5 protein. We also examined Nile Red staining in transgenic animals, which overexpress the K09C4.5::GFP transgene. Even when no drug is present, these animals exhibited significantly reduced Nile Red staining, especially in the anterior and posterior portions of the intestine, which express the transgene most strongly (figure 4-4). This demonstrates that overexpression of the transgene alone is sufficient to alter Nile Red staining, and combined with the finding that drug treatment leads to increased expression of K09C4.5, suggests that the main cause of reduced Nile Red staining in LY294002 treated animals is likely increased function of K09C4.5.

We also examined the subcellular localization of the K09C4.5::GFP protein. We found that the fusion protein was localized almost exclusively to intracellular structures. By crossing K09C4.5::GFP transgenic animals to lines that express either RFP::RAB-5 or RFP::RAB-7, we found that K09C4.5 localization partially overlaps with RAB-7, but minimally overlaps with RAB-5 (figure 4-6). These data strongly suggest that K09C4.5 traffics through the endocytic pathway, with a significant portion of the protein localized to late endocytic structures. We also found that K09C4.5::GFP localizes to the membrane of Nile Red staining gut granules (figure 4-6). In cells expressing the transgene, all Nile Red granules are ringed by K09C4.5::GFP, however a significant amount of K09C4.5::GFP is associated with smaller intracellular puncta that do not stain with Nile Red. The colocalization of this protein with the Nile Red staining structure is compelling, and indicates K09C4.5 function may be integral to the function of this organelle, a hypothesis that warrants further investigation.

One of only a few well-characterized proteins that localizes to the membrane of *C. elegans* gut granules is PGP-2. Loss of this protein leads to a near complete disappearance of Nile Red staining (Schroeder et al., 2007). In addition, animals lack gut autofluorescence, and staining with acridine orange, a marker of acidic organelles, was likewise nearly completely lacking. Hermann and colleagues suggest that these changes indicate that gut granules fail to form in *pgp-2* mutant animals (Schroeder et al., 2007). The lack of gut granule Nile Red staining in *pgp-2* mutant animals is reminiscent of the low Nile Red staining caused by treatment with LY294002. We therefore asked whether loss of K09C4.5 could suppress *pgp-2* mutants as it does the reduced Nile Red staining in drug treated animals. We generated *pgp-2(gk114); (ft6)* double mutant animals, but

found that loss of K09C4.5 fails to suppress the low Nile Red staining in a *pgp-2* mutant background (figure 4-8). If PGP-2 is required for biogenesis of gut granules, and K09C4.5::GFP localizes to these gut granules, we suspected that in *pgp-2* mutant animals K09C4.5 would be mislocalized. We found in fact that while K09C4.5::GFP was abnormally localized to the cell membrane in embryos, in post-embryonic animals, K09C4.5::GFP fusion protein continued to localize to intestinal granules, approximately the size and shape of normal gut granules in *pgp-2* mutant animals (figure 4-9). These data indicate that either *pgp-2* mutants have gut granules that merely fail to fill with the Nile Red staining, autofluorescent material, or possible that K09C4.5 is mislocalized to another intestinal compartment in *pgp-2* mutant animals. To further investigate this question, additional markers of intestinal organelles are required.

Beyond characterizing *ft6* mutants and the K09C4.5 protein, we sought to identify other genes that may function with K09C4.5. We reasoned that such genes might have been identified in a large-scale RNAi screen for Nile Red phenotypes (Ashrafi et al., 2003). Through screens with genes identified in this study, we found that *ft6* suppresses the reduced Nile Red staining caused by RNAi mediated knockdown of *rab-7* (figure 4-10). RAB-7 is a component of the endocytic pathway, and has roles in maturation of endocytic compartments (Vanlandingham and Ceresa, 2009). In addition, RAB-7 has been found to be involved in recycling of growth factor receptors and nutrient transporters, processes that require the endocytic pathway (Edinger et al., 2003; Vanlandingham and Ceresa, 2009). Beyond the finding that *ft6* suppresses the Nile Red reduction caused by RNAi of *rab-7*, two other links between *rab-7* and the K09C4.5 pathway exist. First, RAB-7 and K09C4.5::GFP partially colocalize. Second, RAB-7

has been shown to form a complex with VPS-34, a class III PI3 kinase (Stein et al., 2005; Stein et al., 2003). Based on this relationship, and the fact that LY294002 inhibits mammalian VPS34 (Knight et al., 2006), we tested the ability of this drug to affect FYVE::GFP localization in *C. elegans*, a reporter of VPS-34 function (Roggo et al., 2002). We found that drug treatment indeed inhibited localization of the reporter protein (figure 4-11), demonstrating LY294002 likely inhibits the function of VPS-34 and therefore also RAB-7 function. Initial observations indicated that RNAi mediated knockdown of *rab-7* increases GFP fluorescence in KQ545 animals expressing the K09C4.5::GFP transgene. This would seem to indicate that RAB-7 has a role in degradation of K09C4.5, as has been shown for other nutrient transporters in mammalian cells (Edinger et al., 2003). Therefore, LY294002 may act through two mechanisms to upregulate K09C4.5 function. We have clearly demonstrated that drug treatment induces expression of K09C4.5. Our identification of *rab-7* as a component of the pathway, coupled with the findings that LY294002 inhibits VPS-34 *in vivo* and knockdown of *rab-7* leads to increased K09C4.5::GFP levels strongly suggest that drug treatment also reduces degradation K09C4.5.

These studies have revealed the outline of a genetic network regulating Nile Red staining and gut granule biology. We have shown that LY294002 treatment significantly reduces Nile Red staining, BODIPY-fatty acid staining and gut granule autofluorescence. Furthermore we identified K09C4.5 as gene required for these effects of LY294002, and have demonstrated a mechanism by which LY294002 regulates K09C4.5. We have characterized K09C4.5 expression pattern and subcellular localization. In addition, we found that the Nile Red reduction caused by knockdown of *rab-7* also requires K09C4.5,

and uncovered evidence suggesting LY294002 inhibits RAB-7 function, likely affecting K09C4.5 protein turnover.

A number of important questions remain regarding this pathway. These will have to be investigated before we have a clear understanding of the physiological role this pathway plays. We initially began working on this pathway based on the assumption that the reduced Nile Red staining caused by LY294002 was an indication of a reduced total fat content. However, this appears not to be the case. We measured total TAG levels using TLC, and found that within the detection limit of TLC, there is no significant difference between LY294002 and DMSO control treated animals. Through optimization of this assay, and inclusion of 9 replicates of each condition tested, it is likely we would have been able to detect a change as small as 10-20%. What the change in Nile Red staining indicates remains unresolved. Clearly gut granules are affected by LY294002 treatment, as both Nile Red staining and autofluorescence of these structures is reduced. These findings appear to indicate that the content of these structures has changed. In addition, K09C4.5 localizes to gut granules, further implicating this organelle in the pathway. In order to understand this pathway, we need a better understanding of gut granules. What do they contain and what is their physiological function? A second important question; what does K09C4.5 transport, and how is this affecting gut granule biology? Based on its sequence, K09C4.5 is predicted to function as a sugar transporter, but whether it in fact transports a sugar, and if so which sugar it transports remain to be investigated. Is it transporting sugars into, or out of gut granules? Or might it have a role on the cell membrane? Does it utilize a proton gradient, like HMIT, or does it merely allow a sugar to cross a membrane down its concentration gradient like GLUTs 1, 2 and

4? Whatever its substrate, and wherever it functions within a cell, how is this function linked to gut granule biology?

Future Directions

Many of the experiments described in this study examine Nile Red staining intensity as readout of the physiological consequences of manipulation of the K09C4.5 pathway. While dye staining clearly indicates that a change has taken place, it is unclear what aspect of physiology has changed in these experiments. Better characterization of the Nile Red compartment, and additional means of monitoring this compartment during experimental manipulation would be helpful. A few approaches could be utilized to accomplish this.

A direct method of monitoring the fate and characteristics of this compartment would involve generating a number of protein markers of gut granules as well as other endocytic and lysosomal structures. With good markers, the effects of LY294002 on these organelles could be assessed. In addition, by crossing strains expressing these markers with animals expressing K09C4.5::GFP, it would be possible to determine if drug treatment was altering K09C4.5 localization, as well as whether loss of genes involved in gut granule formation or function affect K09C4.5 localization.

Ideally one would ascertain the affects of LY294002 on subcellular compartments and K09C4.5 localization in time-lapse experiments. Treatment of animals with drug, followed by imaging over a period of hours could reveal disruptions of endocytic trafficking, or loss of particular endosomal structures. These disruptions could be the

result of reduced formation of vesicles, reduced transit of these structures through the endocytic pathway, or inappropriate fusion of different compartments.

Perhaps more challenging, but potentially more informative, would be establishment of a cell culture system. *C. elegans* embryos can be dissociated, and these embryonic cells differentiate into a number of cell types, including intestinal cells (Bianchi and Driscoll, 2006). Cultured intestinal cells are reported to polarize appropriately, and express intestine specific markers, indicating they would likely be useful in studying intestinal processes. Cells could be cultured from animals expressing various markers to allow one to monitor subcellular structures. Ideally cells with multiple markers would be used, which would permit one to watch maturation of endocytic compartments, and fusion of different compartments. Use of cultured intestinal cells would allow for rapid delivery of LY294002, and time lapse imaging. Additionally, cultured cells could be imaged with much better resolution, as diffraction from the cuticle, hypodermis, muscle and gonad would be avoided. Finally, experiments could be conducted for many hours; while at the same time cells could be provided with sufficient nutrients, an advantage over imaging of intact animals.

Another set of experiments would be possible if gut granules could be purified. Though development of this technique would require a significant investment of time, generation of purified gut granules would help shed light not only on the K09C4.5 pathway, but also more generally on the function of this organelle in lipid metabolism. Purification of gut granules, followed by a proteomics analysis would reveal additional markers for this compartment, and may provide clues regarding its function. Analysis of the lipid content of these structures would also help illuminate the link between gut

granules and lipid metabolism. Once a protocol was established, comparison of gut granules from untreated and drug treated wild type and *ft6* mutant animals could reveal how these structures are affected by LY294002. It is likely that the loss of Nile Red staining and autofluorescence indicates a change in the content of gut granules. The most straightforward means to identify the contents of these structures, and how the contents change with drug treatment would be through analysis of purified gut granules.

Identification of the substrate of K09C4.5 could also be helpful. Little is known about intracellular sugar transporters, and if K09C4.5 indeed transports sugar, it could reveal a new physiological role for sugar transport. The identity of the sugar substrate could help eliminate some possible functions of the pathway, and increase the likelihood of others. For example, if the protein transports a “nutrative” sugar such as glucose or fructose, altered K09C4.5 activity may directly be affecting bulk energy distribution, which may indirectly alter gut granule biology. Another group of possible substrates are sialic acids, which if proved substrate for K09C4.5 would indicate disruption of glycolipid metabolism. If mannose proved to be a substrate, K09C4.5 might affect the mannose-phosphate lysosome protein-targeting pathway. Finally, *myo*-inositol appears to be a good candidate substrate, and if proved to be a substrate could indicate that K09C4.5 functions to modulate phosphoinositide signaling, perhaps as a feedback response to inhibition of PI3 kinases.

We attempted two initial approaches to identify potential substrates. Expression of K09C4.5 in hexose uptake deficient yeast failed to rescue uptake, possibly because the protein was targeted to the vacuole, and therefore could not function to take up environmental sugars. We also attempted to purify vacuoles from these yeast, to assay

vacuolar sugar uptake using radiolabeled sugars as has been previously described (Aluri and Büttner, 2007). Unfortunately, we failed to effectively purify yeast vacuoles, a difficulty which should be possible to overcome with optimization of the protocol. Such an approach will overcome the main difficulty in biochemically assessing K09C4.5 transport function, K09C4.5 intracellular localization. An alternative approach, one that we only began to investigate, would be to introduce mutations that prevent internalization of the protein, or force the transporter to the cell membrane. Successful cell membrane targeting of the protein would allow for expression in *Xenopus* oocytes or a mammalian cell culture system, both of which have frequently been used to assay sugar transport (Burant et al., 1992; Ibberson et al., 2000; Wieczorke et al., 2003).

A possibility we have not investigated, but certainly warrants investigation, is a potential connection between the K09C4.5 pathway and autophagy. A number of potential connections exist between these pathways. Autophagosomes are acidic compartments, linked to the endocytic pathway, similar to the compartment to which K09C4.5 localizes. In addition, mammalian RAB7 has been shown to be involved in the maturation of autophagosomes (Gutierrez et al., 2004; Jäger et al., 2004), while we have shown *rab-7* functions in the K09C4.5 pathway. Furthermore, PI3 kinases regulate autophagy via at least two distinct mechanisms. Class I PI3 kinases, generally acting downstream of growth factor receptors, act to inhibit autophagy, while the class III PI3 kinase VPS34 is required for autophagy (Petiot et al., 2000). We have shown that K09C4.5 is regulated by PI3 kinase signaling, by the class I PI3 kinase *age-1*, and possibly by the class III *vps-34* as well. Further supporting a link between these pathways, LY294002, the drug used for many of our studies is a potent inhibitor of

autophagy (Petiot et al., 2000), (though in certain circumstances it may act as an activator of autophagy). Finally, a recent report has described an important role for basal autophagy in lipid metabolism (Singh et al., 2009). Considering the numerous links between the pathway we have described here, and the autophagy pathway, additional investigation would appear to be called for.

Materials and methods

Strains and plasmids used in this study

Strain	Genotype
CF1041	daf-2(e1370) III
FR480	swEX312 [rol-6 (su1006) pRF4 + hsp16-2::GFP::2xT10G3.5 (FYVE)]
KQ27	K09C4.5(ft6)
KQ134	daf-16(mgDf47) I.
KQ193	daf-16(mgDf47) I; daf-2(e1370)
KQ383	N2; ftEX208 [pK09C4.5::GFP line 1]
KQ384	N2; ftEX208 [pK09C4.5::GFP line 3]
KQ385	N2; ftEX208 [pK09C4.5::GFP line 10]
KQ526	K09C4.5(ft6); ftEX261 [pK09C4.5(4.5kb)::K09C4.5gDNA::GFP; Podr-1::RFP]
KQ527	K09C4.5(ft6); ftEX262 [pK09C4.5(4.5kb)::K09C4.5gDNA::GFP; Podr-1::RFP]
KQ543	unc-119 (ed3) ftEX275 [pK09(4.6kb)::K09C4.5gDNA::GFP]
KQ545	K09C4.5(ft6); ftIS21 [pK09(4.6kb)::K09C4.5gDNA::GFP]
KQ546	N2; ftIS21 [pK09(4.6kb)::K09C4.5gDNA::GFP, odr-1::dsRed]; pwIS421 [vha-6::dsRed::rab-7]
KQ547	N2; ftIS21 [pK09(4.6kb)::K09C4.5gDNA::GFP, odr-1::dsRed]; pwIS421 [vha-6::dsRed::rab-7]
KQ548	N2; ftIS21 [pK09(4.6kb)::K09C4.5gDNA::GFP, odr-1::dsRed]; pwIS480 [vha-6::dsRed::rab-5]
KQ549	N2; ftIS21 [pK09(4.6kb)::K09C4.5gDNA::GFP, odr-1::dsRed]; pwIS480 [vha-6::dsRed::rab-5]
KQ1877	K09C4.5 (ft6); pgp-2 (gk114) line 28
KQ1884	pgp-2(gk114) ftIS21[pK09(4.6kb)::K09C4.5gDNA::GFP; odr-1::RFP]
RT1103	unc-119; pwIS421 [vha-6::RFP::rab-7 with let-858 3'UTR]
RT1239	unc-119; pwIS480 [vha-6::RFP::rab-5 with let-858 3'UTR]
VC134	pgp-2(gk114)

Plasmid	
pKA169	K09C4.5 cDNA isolate 1 from N2 TA cloned into pCR2.1 TOPO
pKA170	K09C4.5 cDNA isolate 2 from N2 TA cloned into pCR2.1 TOPO
pKA171	K09C4.5 cDNA isolate 3 from N2 TA cloned into pCR2.1 TOPO
pKA172	K09C4.5 cDNA isolate 4 from N2 TA cloned into pCR2.1 TOPO
pKA173	K09C4.5 cDNA isolate 1 from K09C4.5(ft6) TA cloned into pCR2.1 TOPO
pKA174	K09C4.5 cDNA isolate 2 from K09C4.5(ft6) TA cloned into pCR2.1 TOPO
pKA175	K09C4.5 cDNA isolate 3 from K09C4.5(ft6) TA cloned into pCR2.1 TOPO
pKA176	K09C4.5 cDNA isolate 4 from K09C4.5(ft6) TA cloned into pCR2.1 TOPO
pKA447	yeast expression plasmid containing ADH promoter
pKA555	yeast ADH promoter::K09C4.5cDNA::mCherry for yeast expression
pKA1069	pVha-6::Lmp-1(cDNA)::mCherry
pKA1085	pVha-6::GFP::mutant pgp-2 cDNA (from Greg Hermann)
pKA1148	yeast ADH promoter::K09C4.5cDNA(L 13,14 mut to A)::mCherry for yeast expression
pKA1149	yeast ADH promoter::K09C4.5cDNA(L 28,29 mut to A)::mCherry for yeast expression
pKA1158	yeast ADH promoter::K09C4.5cDNA(L 13,14,17,18 mut to A)::mCherry for yeast expression
pKA1159	yeast ADH promoter::K09C4.5cDNA(L 13,14,28,29 mut to A)::mCherry for yeast expression
pKA1160	yeast ADH promoter::K09C4.5cDNA(L 13,14,17,18,28,29 mut to A)::mCherry for yeast expression
pKA1173	pVha-6::mCherry::mutant pgp-2 cDNA

Nile Red staining and quantification

To make Nile Red plates, 1 μ l of 500 μ M stock (stock made in Acetone) was diluted into 300 μ l of dH₂O for each plate, and surface applied to OP50 seeded 6 cm plates. These plates were allowed to dry for at least an hour prior to applying animals. For most experiments, synchronized L1 animals were applied and allowed to grow for 40-72 hours to the L4 or adult stage before imaging. To quantify Nile Red intensity, animals were imaged with the 5x objective using an exposure that did not lead to saturation of any pixels. A circular region of interest large enough to encompass an animal in any orientation was applied to the image of each animal. The minimum fluorescence was subtracted from the mean fluorescence to remove background signal. Generally 6-10 animals in each experimental group were imaged for each assay.

BODIPY staining

BODIPY-fatty acids were prepared by diluting 0.2 μ l of 1mg/ml C1-BODIPY 500/512 C12 stock in DMSO into 300 μ l of dH₂O for each plate, and this was surface applied to 6cm plates seeded with OP50. This produced a final dilution of 1:50,000 from the original 1mg/ml BODIPY stock. These plates were allowed to dry, and synchronized L1 animals were plated and allowed to grow for 2 days to the young adult stage.

Lysotracker staining

Plates were prepared with 2 μ M Lysotracker Red final concentration. Synchronized L1 animals were plated, and allowed to grow for 40 hours at 20° C. Before imaging, animals were picked to plates without stain, and allowed to feed in order to clear the gut for 1-2 hours.

LY294002 treatment

LY294002 stock solution was prepared at 20 mM in DMSO. This was diluted into a final volume of 300 μ l of dH₂O for each plate, at final concentrations ranging from 25 to 100 μ M as indicated. For experiments in which LY294002 treated animals were stained with other dyes, drug was applied to plates already containing dye, and an equal amount of DMSO was applied for vehicle control. Concentrations of LY294002 reported assume equilibration of LY294002 concentration throughout the entire plate volume (10 ml for 6 cm plates, and 30 ml for 10 cm plates). Small amounts of drug would often precipitate out on the surface of plates, especially for higher concentrations. It is therefore likely that animals may have been exposed to somewhat higher concentrations, as drug may not have been evenly distributed throughout the plate volume.

FYVE::GFP assay

Strain FR480 contains a 2XFYVE domain from T10G3.5 fused to GFP (Roggo et al., 2002). This domain binds PI(3)P, functioning as a reporter for this lipid. To assess the effect of LY294002 treatment on localization of this reporter, FR480 L3 and L4 roller animals were picked onto plates with either DMSO vehicle, or 100 μ M LY294002, and allowed to grow overnight. Transgenic animals were heat shocked for 15 minutes at 37° C, and allowed to recover at room temperature for 2-3 hours. Images were collected with a 60x objective.

Fatty acid oxidation assay

Synchronized L4 animals were washed off plates with 0.9% NaCl, then rinsed three times in 0.9% NaCl. Following washes, animals were incubated for 20 minutes in 0.9% NaCl to allow animals to empty their intestines and then resuspended in S-basal in a final volume of 540 μ l. An aliquot (20 μ l) was stored at –80° C for subsequent protein

determination. [9,10(n)-3H] Oleic acid (GE Healthcare) was added to worms to a final concentration of 20 μ M (specific activity 67-93 Ci/mol), complexed to fatty acid free BSA in a 2:1 molar ratio. Samples were incubated on an orbital shaker for one hour at 20° C. Subsequently, TCA was added to a final concentration of 5% w/v. After mixing, samples were centrifuged (14000xg, 10min) and the supernatant (1ml) was transferred to a new microcentrifuge tubes. Phosphate buffered saline (250 μ l) and 5M NaOH (100 μ l) were added prior to ion exchange chromatography on Dowex 1x8 (200-400 MESH) ion exchange columns (1ml). Samples were eluted using water (1ml) and the amount of ^{3} H₂O in the eluate was determined by scintillation counting. Samples without animals were included as background controls.

Protein determination

For both fatty acid oxidation and TLC experiments, values were normalized to protein content. To determine protein content, 200 μ l of extraction buffer (7M urea, 2M thiourea, 4% CHAPS, 1mM EDTA 50mM HEPES pH7.4, with TCEP added just before use at 5mM) was added to a 50 μ l of worms. This mixture was incubated at 37° C. Protein concentration was determined with Bradford reagent. Generally, 25 to 75 μ l of extract was used for protein determination. This method of protein determination is highly reproducible, and appears to efficiently extract nearly all the protein from animals, leaving behind only empty cuticular shells. Use of this method proved much more reliable than protein determination from samples from which protein was extracted via sonication.

Thin layer chromatography

For each replicate, plate 5,000 L1 animals on a 10 cm plate seeded with OP50. Grow animals for ~40 hours to the L4 stage. It is critical that all animals across conditions are precisely staged. Rinse animals from each plate into a 15 ml conical tube with S-Basal containing 0.01% PEG. Rinse 3X in 10 ml S-Basal with PEG to remove bacteria. After the final wash, pellet animals and aspirate off most of the supernatant, leaving ~1 ml behind. Pipet 150 μ l from the bottom of each tube (making sure to draw up all the worms) into a 1.5 ml tube. Transfer 50 μ l of this into a second 1.5 ml tube for protein determination, and freeze at -80° C. Tubes with the remaining 100 μ l can be stored at -80° C at this point. To extract lipids, add 59 μ l of dH₂O, 200 μ l chloroform and 400 μ l methanol to each tube. Rotate at room temperature for 20 minutes. Next add another 200 μ l chloroform, 200 μ l of methanol and 200 μ l 0.2 M HCl, and rotate for another 20 minutes at room temperature. Spin at max speed in a desktop centrifuge for 5 minutes, and recover the bottom phase to a fresh 1.5 ml tube. Add 750 μ l “clean supernatant” (to make, combine methanol, chloroform, dH₂O and 0.2M HCl in the same ratios used for extraction of lipids, spin for 5 minutes and recover the top phase). Incubate at room temperature for 20 minutes rotating. Spin at max speed in desktop centrifuge for 5 minutes, and recover the bottom phase to a new 1.5 ml tube. Dry in a SpeedVac, and resuspend in 50 μ l 1:1 methanol:chloroform.

To visualize lipids using TLC, load 5 μ l per sample (this volume may need to be adjusted in order to load 1-3 μ g total TAG, a range over which phosphomolybdic acid stain is sensitive). For each 20 cm plate, leave 2 cm margins to prevent excessive smiling, and load samples every 1 cm (17 lanes per plate). Load standards of 0.5, 1, 1.5, 3 and 6 μ g TAG to generate a standard curve. TLC plates are developed using hexane,

diethyl ether and acetic acid in a 70:30:1 ratio. Allow plates to dry completely, and then spray with 20% phosphomolybdic acid in ethanol. Once the stain has dried, place plates in a 100° C oven for 10 minutes to allow stain to develop. Capture an image of the plate using a scanner, and quantify the density of the TAG spots for each lane. Fit a curve to the standard curve (a 3rd order polynomial generates the best fit) to convert density measurements into TAG quantities. Finally, normalize TAG quantities to protein amounts.

Mutagenesis

Two mutagenesis screens were performed. For both screens, synchronized F2 L1 progeny from mutagenized animals were applied to plates containing 1X Nile Red (50 nM) and LY294002. In the initial screen, 25 µM of drug was used, while in the second screen we used 50 µM to improve selection. For both screens, P0 animals were mutagenized at the early L4 stage using 47 mM EMS (ethyl methanesulfonate) for 4hrs. In the first screen, we assayed progeny from ~11,000 haploid genomes, while in the second screen, ~20,000 haploid genomes were screened.

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Figure Legends

Table 4-1. Putative *C. elegans* sugar transporters

We tested RNAi of this list of putative sugar transporters for suppression of LY204002 induced reduced Nile Red staining. Only RNAi of K09C4.5 suppressed the drug effect.

Table 4-2. A second mutagenesis screen identifies additional suppressors

We performed a second EMS screen for lines that exhibit resistance to LY294002 induced reduction of Nile Red staining. We identified more than 20 new lines. Those labeled in blue exhibit nearly wild type staining, but significantly suppress the Nile Red reduction caused by LY294002 treatment. These may include additional K09C4.5 alleles, but may be the most interesting lines to follow up.

Figure 4-1. LY294002 reduces Nile Red staining

We found that treatment of wild type animals with the broad spectrum PI3 kinase inhibitor LY294002 leads to significantly reduced Nile Red staining. In (A) young adult animals treated with either DMSO vehicle or 100 μ M LY294002 and stained with Nile Red are shown. The structure of LY294002 is shown in (B).

Figure 4-2. A mutagenesis screen identifies *ft6*, a mutant line resistant to the Nile Red reduction caused by LY294002

In order to better understand the mechanism by which LY294002 acts to reduce Nile Red staining, we carried out a forward mutagenesis screen. P0 animals were treated with the mutagen EMS, and synchronized F2 progeny from these mutagenized animals were

screened on plates containing LY294002 and Nile Red (A). From this screen, we identified *ft6*, which is resistant to the Nile Red reducing effect of LY294002 treatment. Images show L4 wild type and *ft6* mutant animals treated with DMSO, 25 μ M or 100 μ M LY294002 (B). Quantification of Nile Red staining intensity is shown in panel (C).

Figure 4-3. Positional cloning and sequence analysis reveals a mutation in K09C4.5 in *ft6* animals

Utilizing SNP linkage analysis, we mapped the *ft6* mutation to a region of the X chromosome between -11.59 and -10.5 map units (A). We found that cosmid K09C4 rescued *ft6* animals. Sequencing of K09C4.5 identified a G to A mutation in the predicted 3' splice site of the 3rd intron (B and D). We cloned K09C4.5 cDNAs from wild type and *ft6* mutant animals, and found that *ft6* cDNAs are misspliced (C).

Figure 4-4. Expression of wild type K09C4.5 rescues *ft6* mutants, and expression is limited to the intestine

To verify that loss of K09C4.5 in fact causes the *ft6* mutant phenotype, we expressed the wild type gDNA with 4.6kb of upstream regulatory sequence, and C-terminally fused to GFP. This construct exhibited clear expression in the intestine, but not in other tissues (A). We treated transgenic animals, and non-transgenic siblings with 100 μ M LY294002 to assess the ability of this construct to rescue the mutant phenotype. In panel (A), a non-transgenic L3 animal is shown above a transgenic young adult. The non-transgenic animal exhibits bright Nile Red staining, though treated with drug. The transgenic sibling exhibits intestinal GFP expression, but very low Nile Red staining, demonstrating

this construct does in fact rescue the *ft6* mutant phenotype (A). In animals not treated with drug, expression of the transgene leads to reduced Nile Red staining. In panel (B), a non-transgenic animal is on the left, and two transgenic animals are to the right. The transgenic animals exhibit reduced Nile Red staining in the anterior and posterior intestine, regions exhibiting the highest transgene expression. Arrows indicate anterior and posterior intestine.

Figure 4-5. Mosaic animals reveal K09C4.5 functions cell autonomously

A small number of KQ526 transgenic animals exhibited mosaic expression. Animals shown were treated with 100 μ M LY294002 and stained with Nile Red. Mosaic expression is clearly evident in an L3 animal (A), in which roughly half the intestinal cells are transgenic. These transgenic cells exhibit the wild type response to LY294002, that is, they stain very little with Nile Red. Non-transgenic cells, which are not labeled with GFP, exhibit bright Nile Red staining. Another example shown in (B) is of an L4 animal. Only one intestinal cell in the image is non-transgenic, and this cell clearly exhibits bright Nile Red staining, even though the animal is treated with drug. These animals demonstrate that K09C4.5 functions cell autonomously. (Non-transgenic intestinal cells exhibit a low amount of green fluorescence due to autofluorescence.)

Figure 4-6. K09C4.5::GFP localizes to Nile Red staining gut granules, and exhibits partial colocalization with endocytic markers

KQ545 transgenic exhibit K09C4.5::GFP localized to Nile Red staining gut granules (A). In an L3 animal, Nile Red staining is shown in (1), K09C4.5::GFP in (2) and the merged

image in (3). K09C4.5 partially colocalizes with RFP::RAB-7, a marker of late endocytic compartments, but colocalizes very little with RFP::RAB-5, a marker of early endosomes (B). Autofluorescence is pseudocolored blue in panel (B).

Figure 4-7. K09C4.5::GFP does not localize to LMP-1::mCherry positive lysosomes

Intestinal gut granules, the structures that stain with Nile Red have been characterized as a lysosome-related structure. To examine the possibility that K09C4.5::GFP localizes to lysosomes as well, we generated a LMP-1::mCherry construct, and expressed it in KQ545 transgenic animals. However, we found no significant overlap in K09C4.5::GFP and LMP-1::mCherry localization.

Figure 4-8. *pgp-2* is epistatic to K09C4.5

Exposure to LY294002 causes reduced Nile Red staining, as does loss of *pgp-2*. To investigate whether K09C4.5 might function downstream of *pgp-2* as it does downstream of LY294002, we generated *pgp-2(gk114); ft6* double mutants (strain KQ1887) and examined their Nile Red staining phenotype. As is clearly evident in this figure, *pgp-2(gk114); ft6* double mutants exhibit the same loss of Nile Red staining phenotype as *pgp-2(gk114)* single mutants, demonstrating that *pgp-2* is epistatic to K09C4.5.

Figure 4-9. Loss of *pgp-2* disrupts K09C4.5::GFP localization

PGP-2 is involved in gut granule formation or function. To examine the effect of loss of *pgp-2* on K09C4.5::GFP localization, we crossed KQ545, expressing K09C4.5::GFP to *pgp-2(gk114)* mutants to generate strain KQ1884. In late stage embryos, the earliest

stage at which K09C4.5::GFP expression is apparent, loss of *pgp-2* clearly caused elimination of nearly all intracellular K09C4.5::GFP labeled vesicles (A). In later stage animals, *pgp-2* mutants exhibited intracellular K09C4.5::GFP labeled vesicles, though these appeared to be somewhat abnormal. An extreme example of disrupted K09C4.5::GFP localization pattern is shown in young adult animals in panel (B).

Figure 4-10. Loss of K09C4.5 suppresses the reduced Nile Red staining caused by RNAi mediated knockdown of *rab-7*

We performed an RNAi screen, using clones previously demonstrated to reduce Nile Red staining, to identify clones that reduce Nile Red in a K09C4.5 dependant manner. We found that RNAi of *rab-7* significantly reduces Nile Red staining in wild type animals, but not *ft6* mutants. L4 wild type and *ft6* mutant animals treated with either vector control or *rab-7* RNAi, and imaged at low magnification are shown in (A). Quantification of Nile Red staining intensity is shown in (B). Data are shown as a percentage of wild type on vector control +/- SEM. *** indicates $p < 0.001$

Figure 4-11. LY294002 disrupts FYVE::GFP localization

Based on the identification of *rab-7* as a in the K09C4.5 pathway, and the fact that this gene functions with the class III PI3 kinase VPS-34, we wished to determine whether LY294002 disrupts VPS-34 function. To test this, we treated animals expressing the PI(3)P binding domain FYVE fused to GFP. Transgenic animals were treated with 100 μ M LY294002 overnight, then heat shocked to induce expression of the reporter.

FYVE::GFP localization was clearly disrupted, as shown in images of the hypodermis in this figure.

Figure 4-12. LY294002 induces expression of K09C4.5

When testing the whether transgenic expression of K09C4.5 could rescue *ft6* mutants, we observed significantly increased K09C4.5::GFP intensity in LY294002 treated animals. To examine this more systematically, we picked synchronized young adult KQ526 transgenic animals, expressing the K09C4.5::GFP transgene, onto plates containing 100 μ M LY294002. We imaged these animals that had been treated for 5 hours with either DMSO vehicle, or 100 μ M LY294002 (A). To test whether increased GFP levels could result from increased expression of K09C4.5 upon drug treatment, we collected RNA from vehicle and drug treated animals, and measured expression of K09C4.5 using RT-PCR. We found that treatment with 50 μ M LY294002 induced 4-fold increased expression of K09C4.5 (B).

Figure 4-13. Genetic inhibition of PI3 kinase signaling induces K09C4.5 expression

Though we had found that LY294002 treatment can induce K09C4.5 expression, we wished to determine whether this could function through inhibition of PI3 kinase signaling. To test this, we compared expression of K09C4.5 in wild type, *daf-16*, *daf-2* and *daf-2; daf-16* animals. DAF-2 functions through activation of the PI3 kinase AGE-1, which in turn functions to inhibit the FOXO transcription factor DAF-16 (A). Therefore, loss of *daf-2* function leads to reduced PI3 kinase signaling. We found that *daf-2* mutants express K09C4.5 at 3.5-fold higher levels than do wild type animals (B). This induced

expression was dependant on *daf-16*. Data are shown as a percentage of wild type +/- SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$

Figure 4-14. LY294002 treatment reduces BODIPY-fatty acid staining and autofluorescence in a K09C4.5 manner

To examine the effect of LY294002 beyond Nile Red staining, we assayed BODIPY-fatty acid staining and autofluorescence in DMSO vehicle and LY294002 treated animals. BODIPY-fatty acid staining was reduced in wild type animals, but this change was dependant on K09C4.5, just as are changes in Nile Red staining (A). Likewise, drug treated animals exhibited reduced levels of autofluorescence, in a K09C4.5 dependant manner (B). Lysotracker staining however was increased in LY294002 treated animals, independent of K09C4.5 (C).

Figure 4-15. LY294002 treatment does not alter TAG levels as assayed by TLC, nor does it alter fatty acid oxidation significantly

The reduced Nile Red staining in LY294002 treated animals could be an indication that total lipid stores are reduced by drug treatment. However, TLC analysis revealed no significant difference between DMSO and LY294002 treated animals, nor between wild type and *ft6* mutant animals (A). We also examined fatty acid oxidation rates by feeding animals radiolabeled oleate and monitoring the production of tritiated water. However, LY294002 treatment did not significantly increase rates of fatty acid oxidation (B). Error bars denote SD.

Table 4-1

***C. elegans* sugar transporters tested**

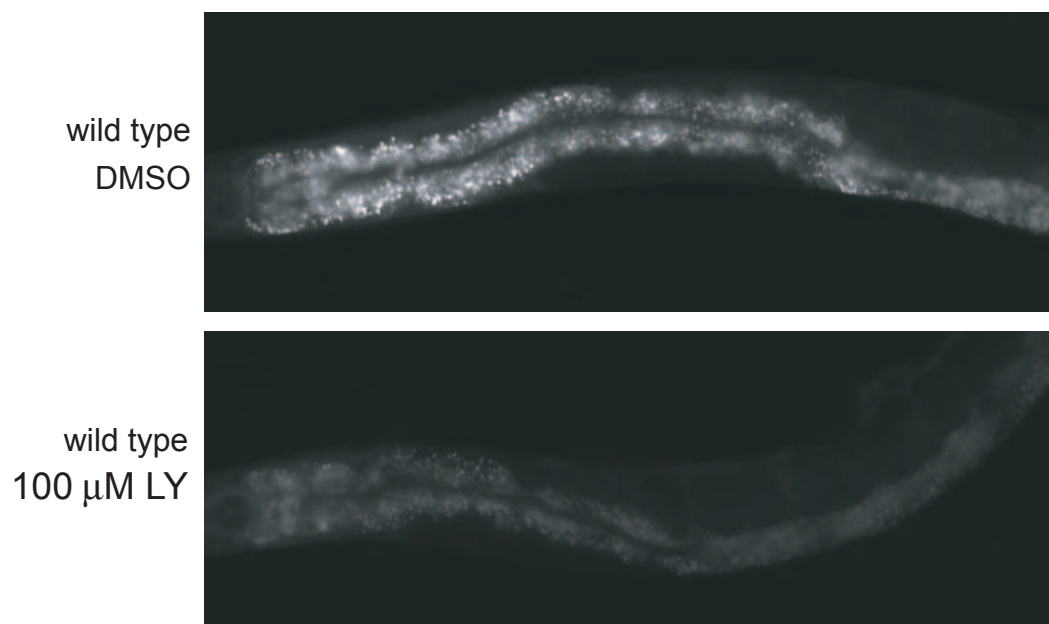
	Gene ID	Ahr Plate	Ahr Well
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2	F14E5.1	51	F7
3	M01F1.5	69	C1
4	Y39E4B.5	88	B10
5	F13B12.2	110	B7
6	ZK829.9	113	F1
7	Y37A1A.3	117	E6
8	R09B5.11	127	G11
9	C35A11.4	137	H7
10	K08H10.6	148	H10
11	K08F9.1	160	F7
12	Y51A2D.4	169	F1
13	Y51A2D.5	169	F2
14	Y61A9LA.1	174	H12
15	F53H8.3	176	H8
16	K09C4.1	180	G3
17	K09C4.2	180	G4
18	K09C4.4	180	G6
19	K09C4.5	180	G7
20	ZK563.1	180	H12
21	F11D5.5	180	H5
22	F48E3.2	187	F6
23	C03B1.13	203	G4

Table 4-2

Strain	Isolate	Reproduction	Growth	Nile Red	Nile Red on LY 50uM	Other phenotypes
KQ2142	SL-G2	slow	slow	patchy, a bit increased	moderate suppression, patchy	a bit small
KQ2143	SL-G3	ok	ok	patchy, a bit increased	moderate suppression, patchy	none
KQ2144	SL-G4	ok	ok/slow	patchy	moderate suppression, patchy	none
KQ2145	SL-G6	ok	ok	~wild type	good suppression	none
KQ2146	SL-G8	ok	ok	patchy, a bit increased	moderate suppression, patchy	a bit small
KQ2147	SL-G9	ok	ok	~wild type	moderate suppression, variable/inpenetrant	
KQ2148	SL-H2	bit slow	bit slow	quite variable	quite variable, some animals may show suppression	100% dumpy
KQ2149	SL-I1	ok	ok	somewhat patchy	moderate/good suppression, a bit patchy	none
KQ2150	SL-J2	ok/slow	slow	patchy, a bit increased	moderate suppression, patchy	none
KQ2151	SL-J3	ok/slow	ok/slow	patchy, a bit increased	moderate suppression, patchy	none
KQ2152	SL-X1	ok	ok	~wild type	moderate/good suppression	none
KQ2153	SL-X3	ok/slow	ok/slow	patchy, a bit increased	moderate suppression, patchy	none
KQ2154	SL-X4	slow	ok/slow	increased, patchy	partial suppression, patchy	none
KQ2155	SL-X5	slow	slow	increased, patchy	partial suppression, patchy	a bit small
KQ2156	SL-X7	ok/slow	ok	increased, patchy	good/moderate suppression	none
KQ2157	SL-X10	ok	ok	~wild type, perhaps a bit hazy?	good suppression	none
KQ2158	SL-X11	slow	ok/slow	a bit increased	good suppression	a bit small
KQ2159	SL-X13	ok/slow	ok	very few, hard to determine	weak suppressor?	none
KQ2160	SL-X14	ok	ok	~wild type	good suppression	none
KQ2161	SL-X15	ok/slow	ok	~wild type	moderate suppression	none
KQ2162	SL-X17	ok/slow	ok/slow	slight increase? Slightly patchy?	good/moderate suppression	small, a bit clear

Figure 4-1

A



B

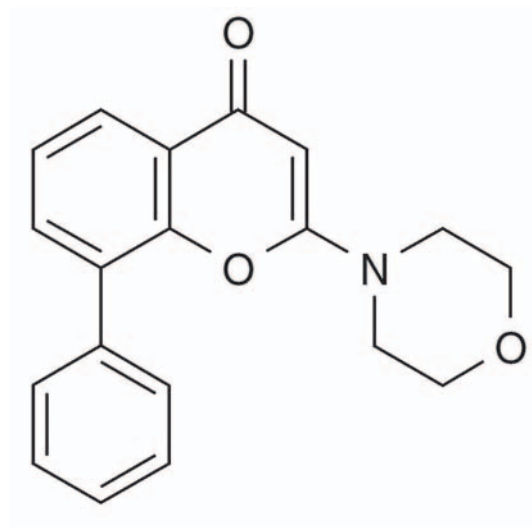


Figure 4-2

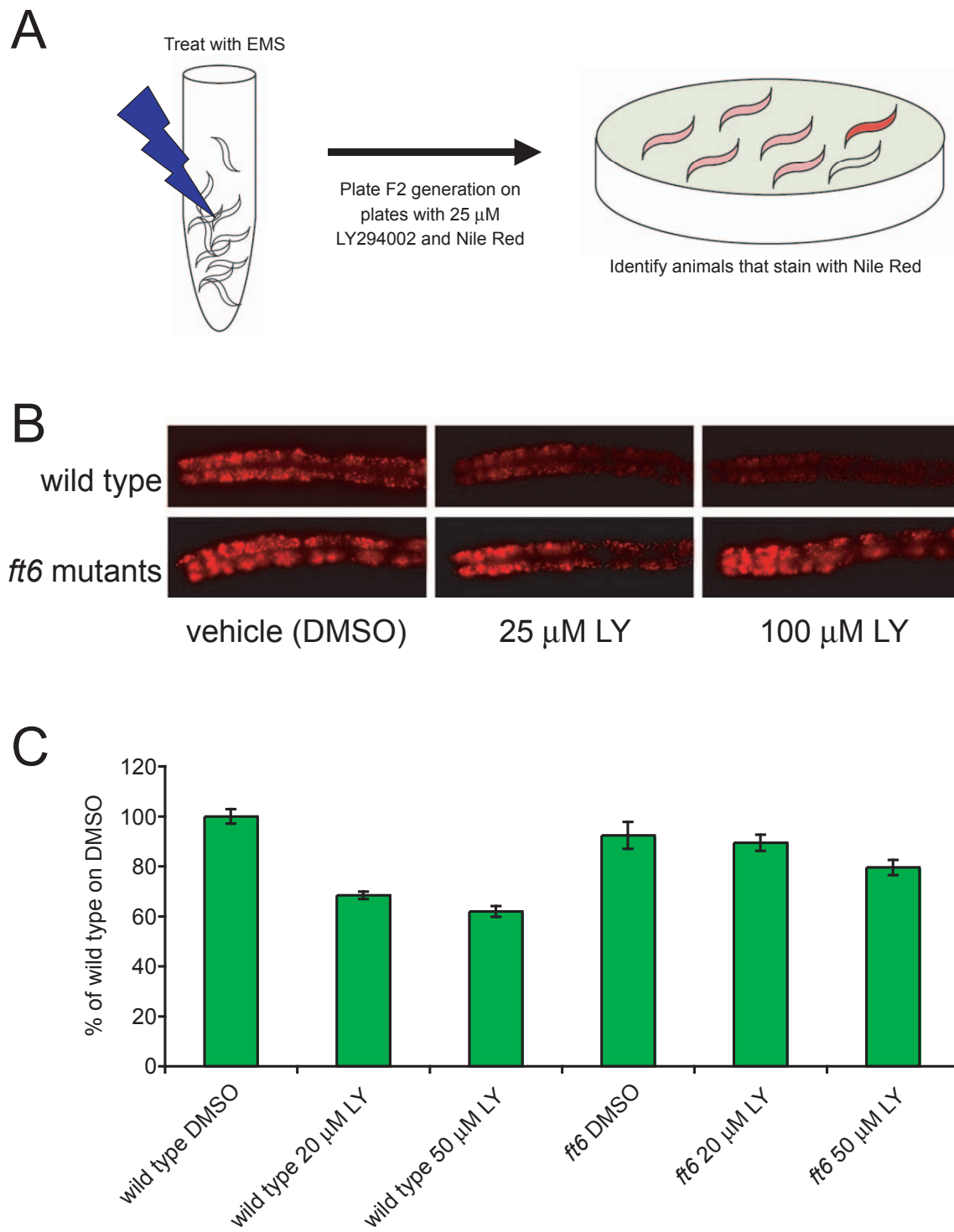
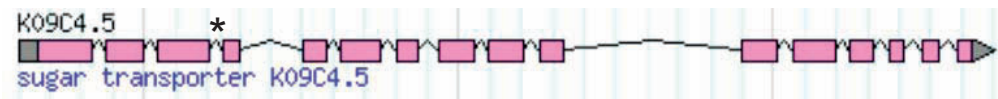


Figure 4-3

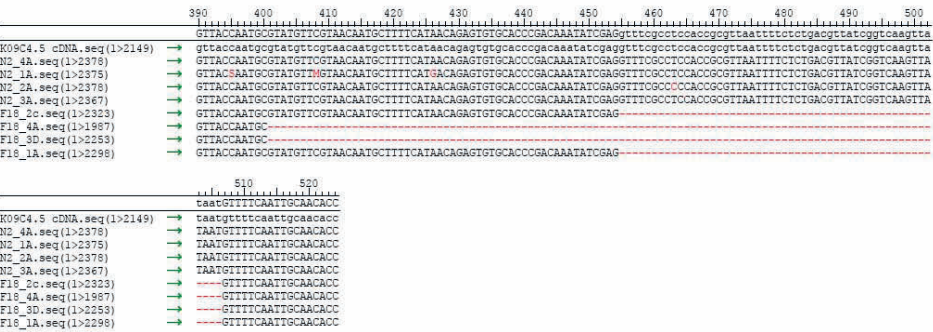
A

Recombinant	pkP6118 (-15.6)	pkP6119 (-12.61)	pkP6145 (-11.59)	pkP6120 (-10.50)	snp_F56B6[1] (-10.14)	pkP6103 (-9.77)	pkP6107 (-6.2)	pkP6110 (-0.8)	pkP6114 (2.86)
4	N2	N2	N2	N2	N2	N2	N2	HET	HET
11	N2	N2	N2	N2	N2	N2	N2	HET	HET
14	N2	N2	N2	N2	N2	N2	N2	HET	HET
18	N2	N2	N2	N2	N2	HET	HET	HET	HET
23	N2	N2	N2	N2	N2	N2	N2	HET	HET
26	N2	N2	N2	N2	N2	N2	N2	HET	HET
29	N2	N2	N2	N2	N2	N2	N2	HET	HET
30	N2	N2	N2	N2	N2	N2	N2	N2	HET
37	N2	N2	N2	N2	N2	N2	N2	HET	HET
39	N2	N2	N2	N2	N2	N2	N2	HET	HET
40	N2	N2	N2	N2	N2	N2	N2	HET	HET
44	N2	N2	N2	N2	N2	N2	N2	HET	HET
45	HET	N2	N2	N2	N2	N2	N2	N2	N2
47	N2	N2	N2	N2	N2	N2	HET	HET	HET
49	N2	N2	N2	N2	N2	N2	N2	N2	HET
50	N2	N2	N2	N2	N2	N2	N2	N2	HET
51	N2	N2	N2	N2	N2	HET	HET	HET	HET
52	N2	N2	N2	N2	N2	N2	N2	N2	HET
53	HET	N2	N2	N2	N2	N2	N2	N2	N2
55	N2	N2	N2	N2	N2	N2	N2	HET	HET
60	N2	N2	N2	N2	HET	HET	HET	HET	HET
62	N2	N2	N2	N2	N2	N2	HET	HET	HET
68	N2	N2	N2	N2	N2	N2	HET	HET	HET
70	HET	N2	N2	N2	N2	N2	N2	N2	N2
216	N2	N2	N2	HET	HET	HET	HET	HET	HET
270	HET	HET	HET	N2	N2	N2	N2	N2	N2
287	HET	HET	HET	N2	N2	N2	N2	N2	N2

B



C



D

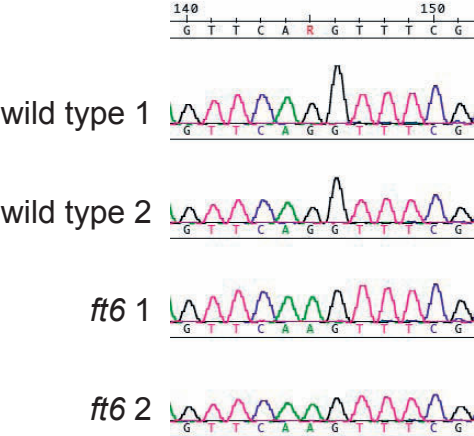


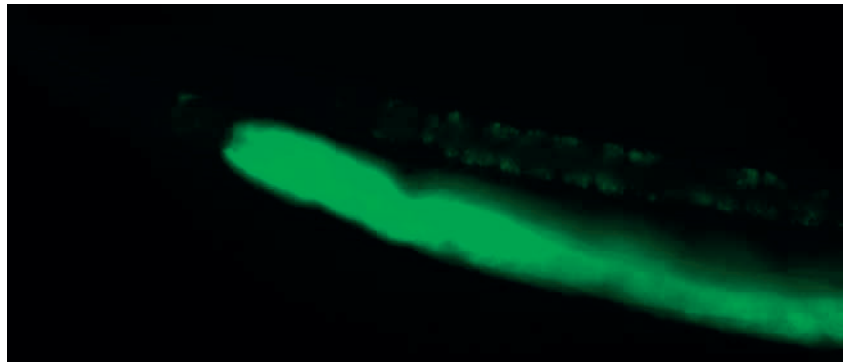
Figure 4-4

A

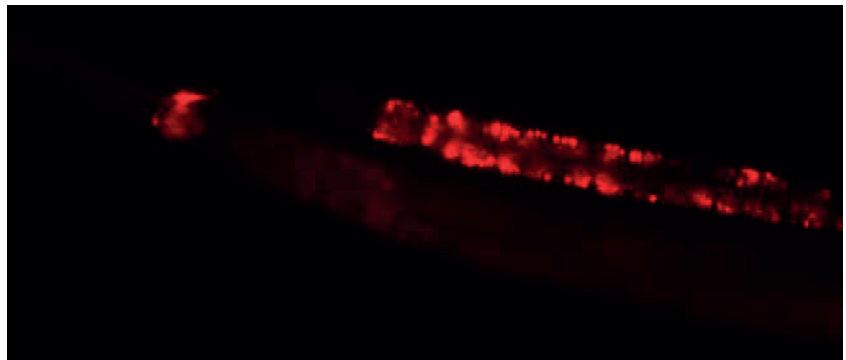
DIC



K09C4.5::GFP

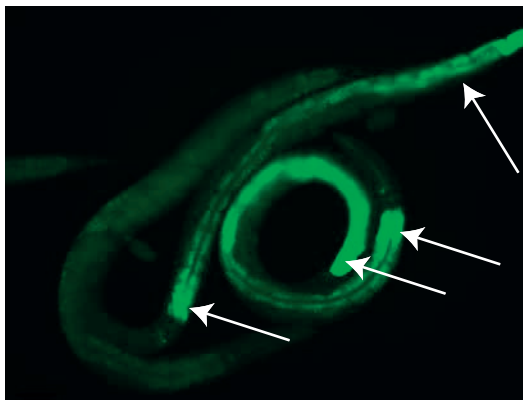


Nile Red



B

K09C4.5::GFP



Nile Red

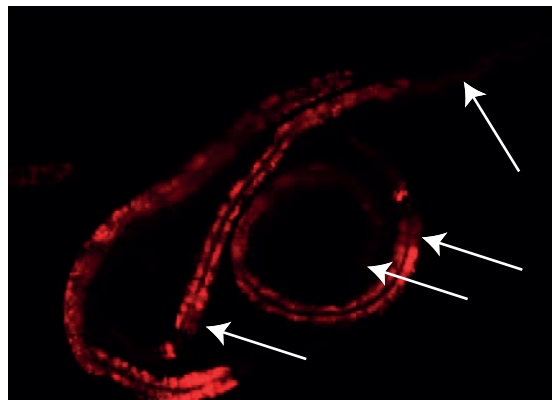
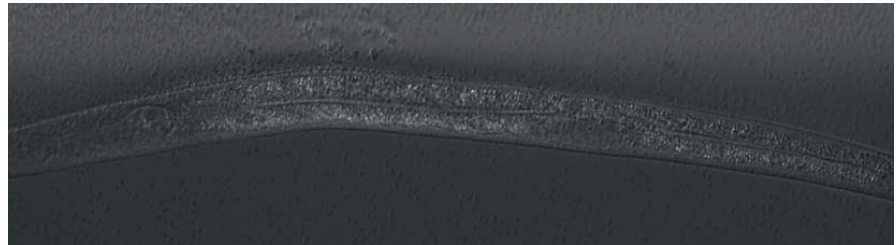


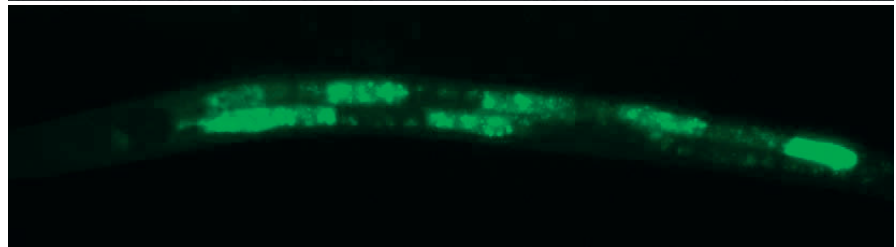
Figure 4-5

A

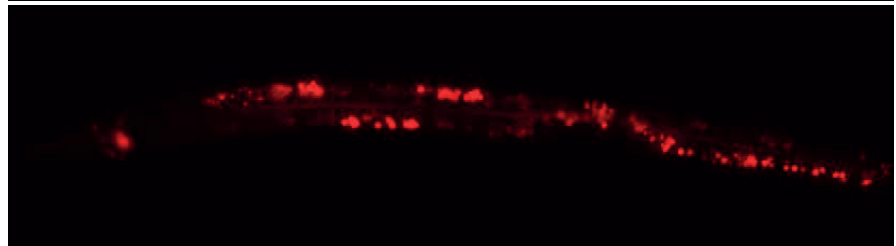
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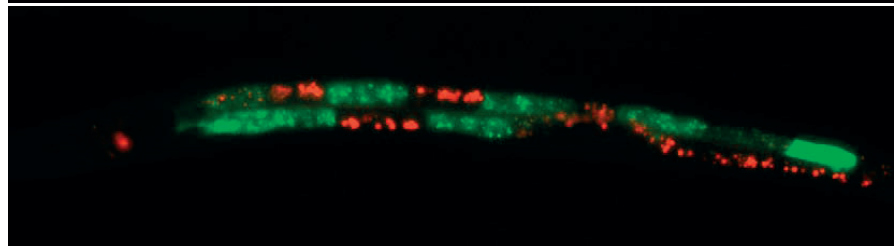
K09C4.5::GFP



Nile Red

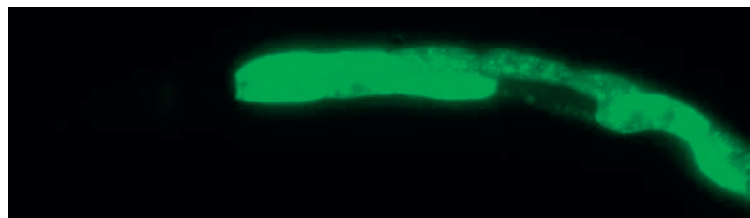


Merge

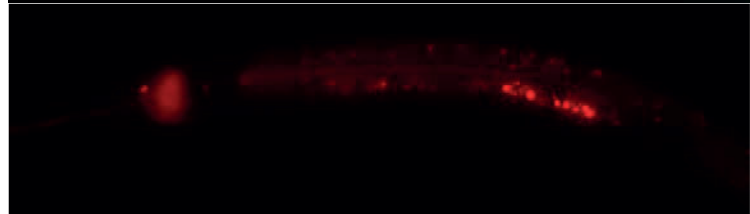


B

K09C4.5::GFP



Nile Red



Merge

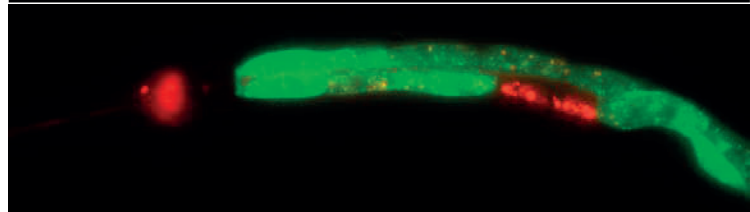
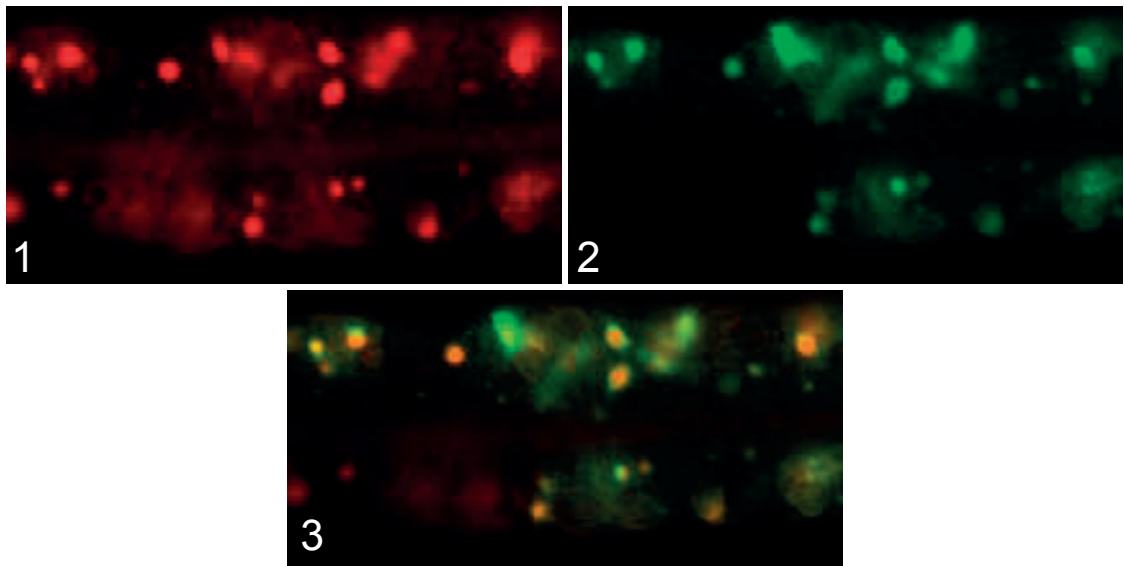


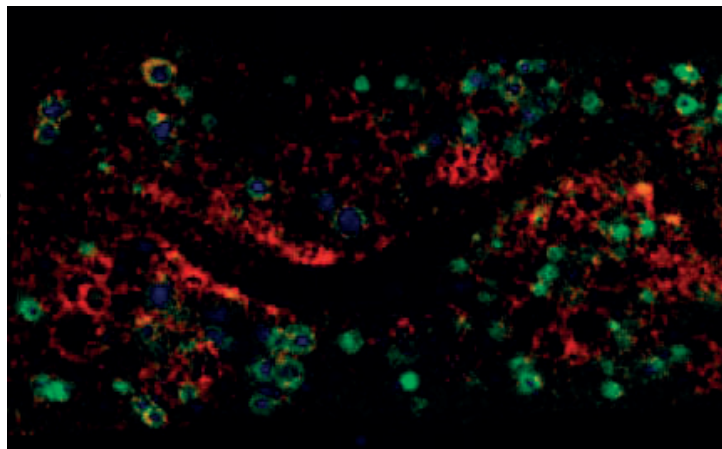
Figure 4-6

A



B

K09C4.5::GFP
RFP::RAB-7



K09C4.5::GFP
RFP::RAB-5

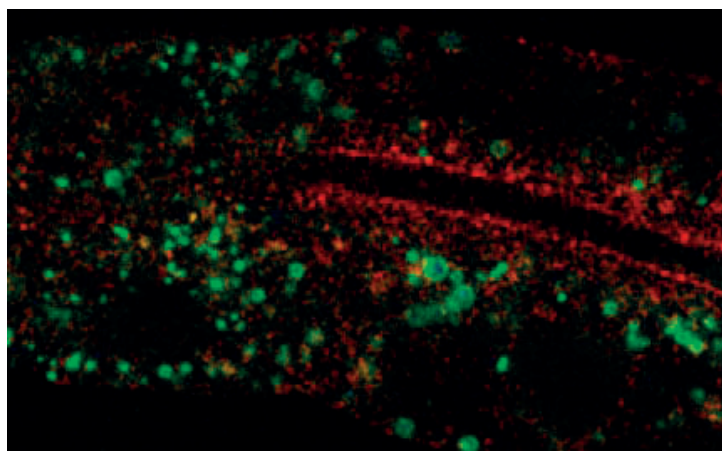


Figure 4-7

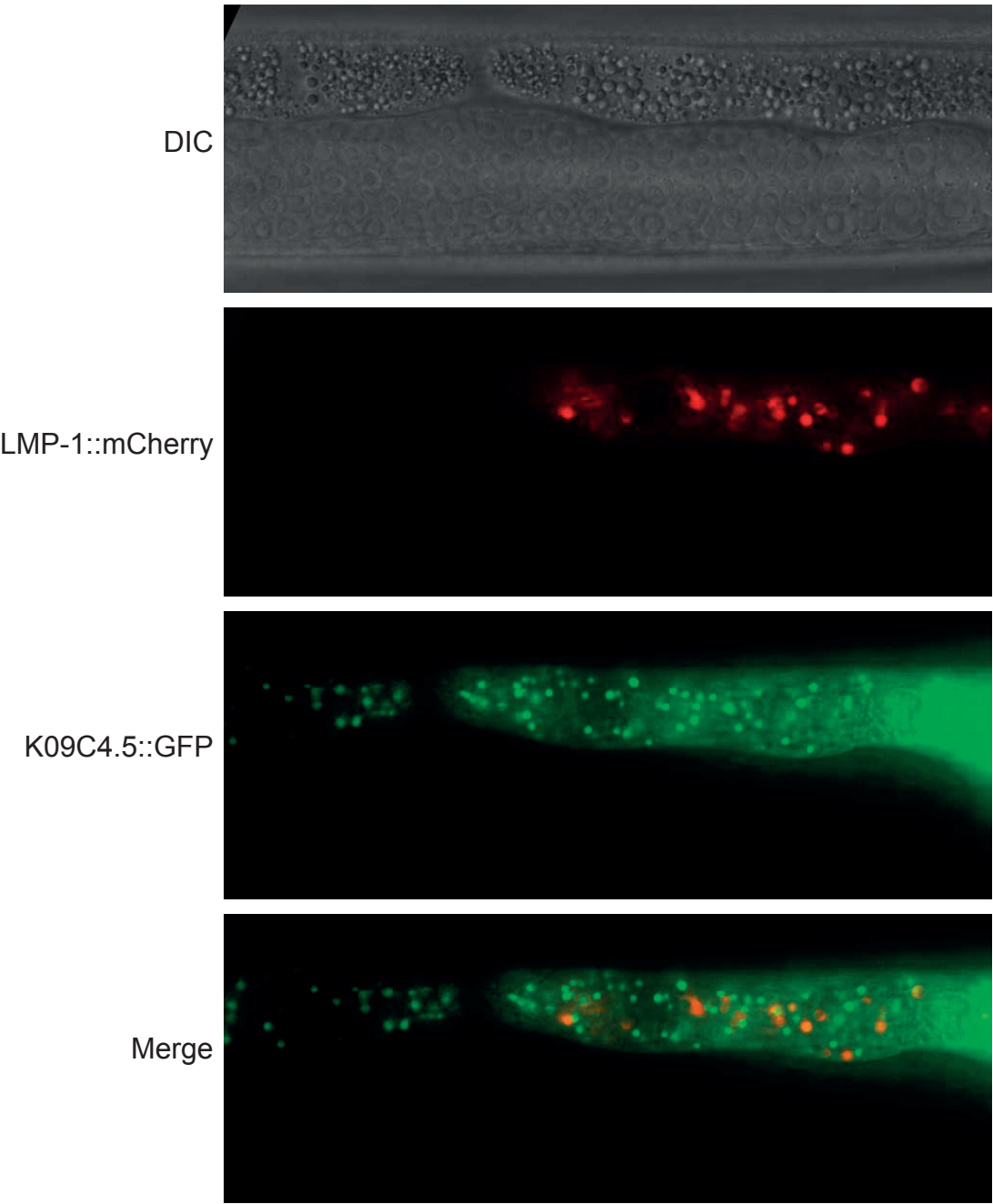


Figure 4-8

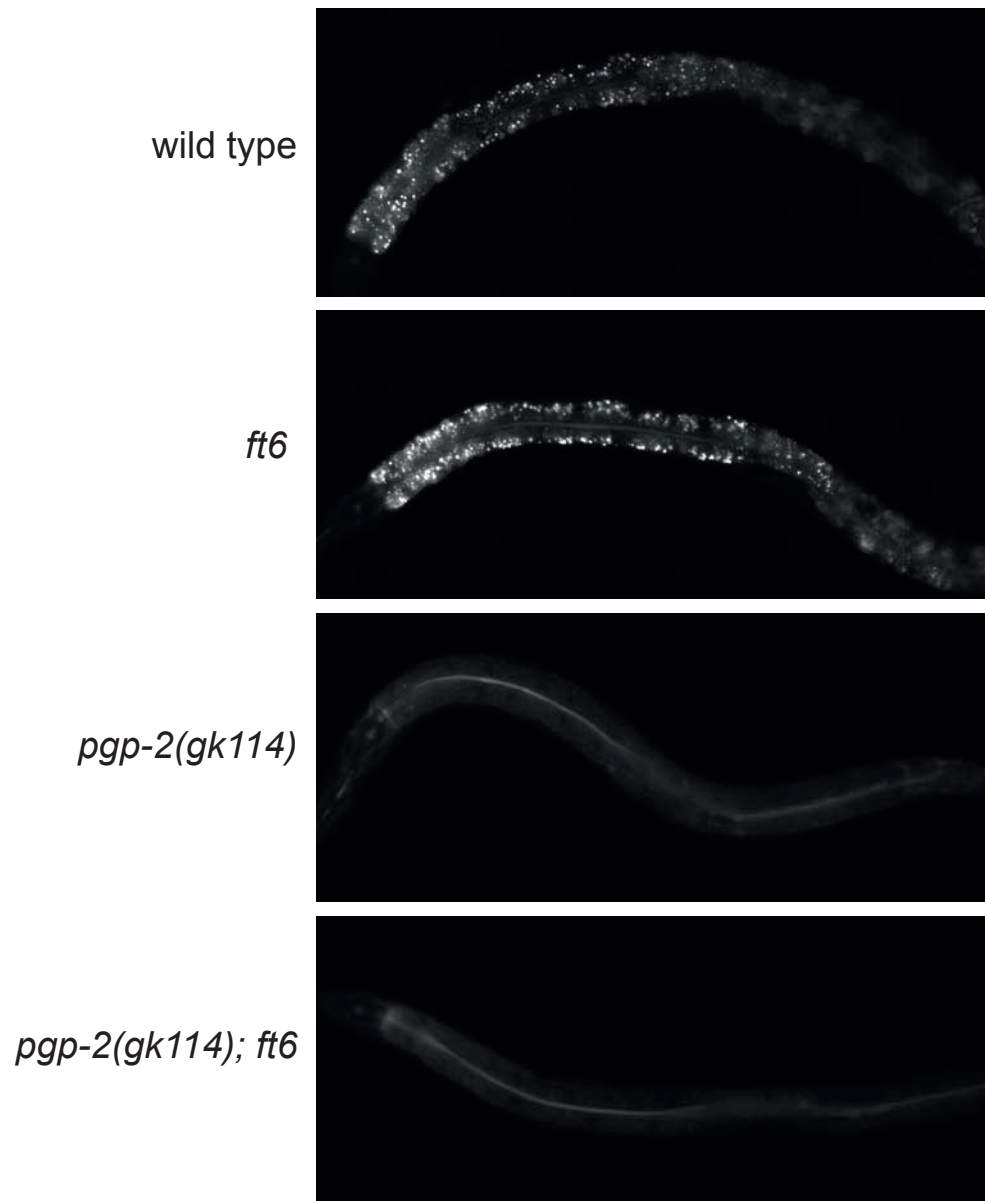
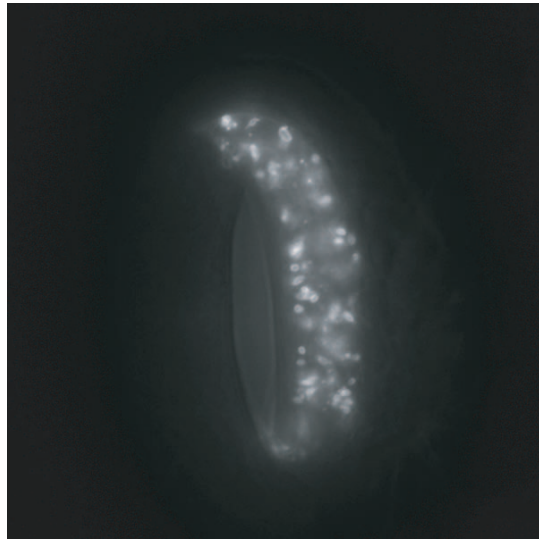
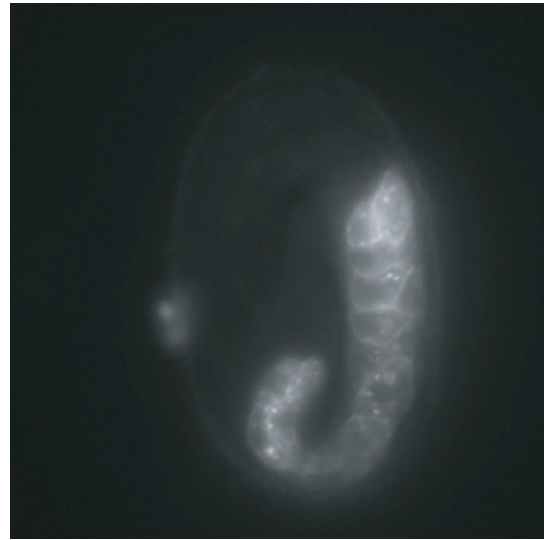


Figure 4-9

A



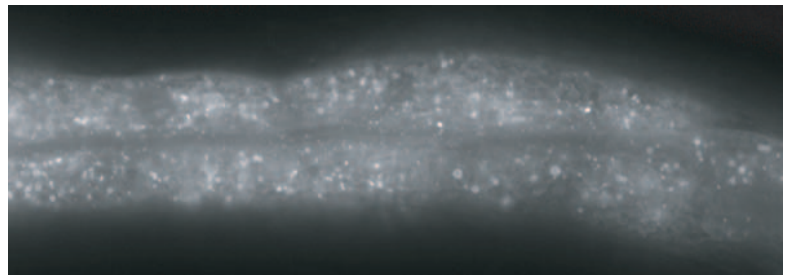
K09C4.5::GFP in wild
type background



K09C4.5::GFP in *pgp-2(gk114)*
background

B

K09C4.5::GFP in wild
type background



K09C4.5::GFP in *pgp-2*
(*gk114*) background

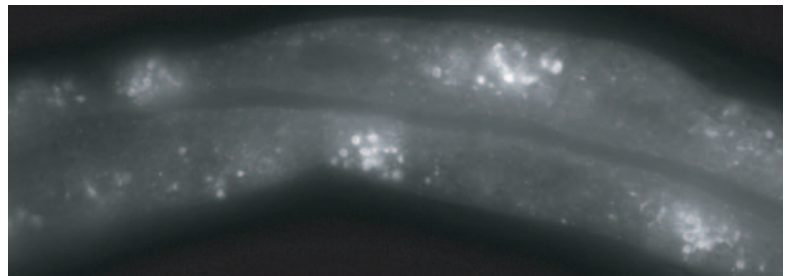


Figure 4-10

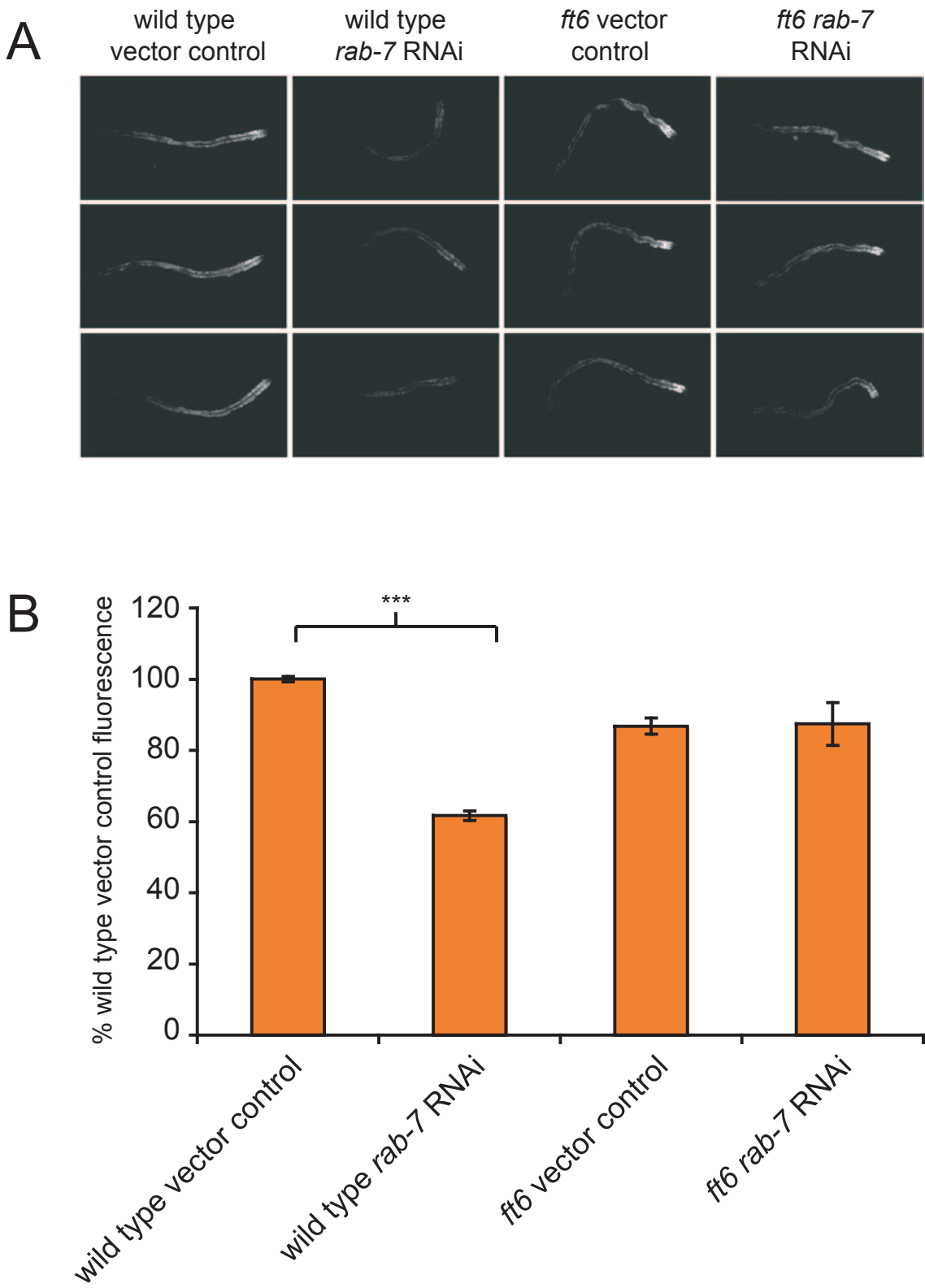


Figure 4-11

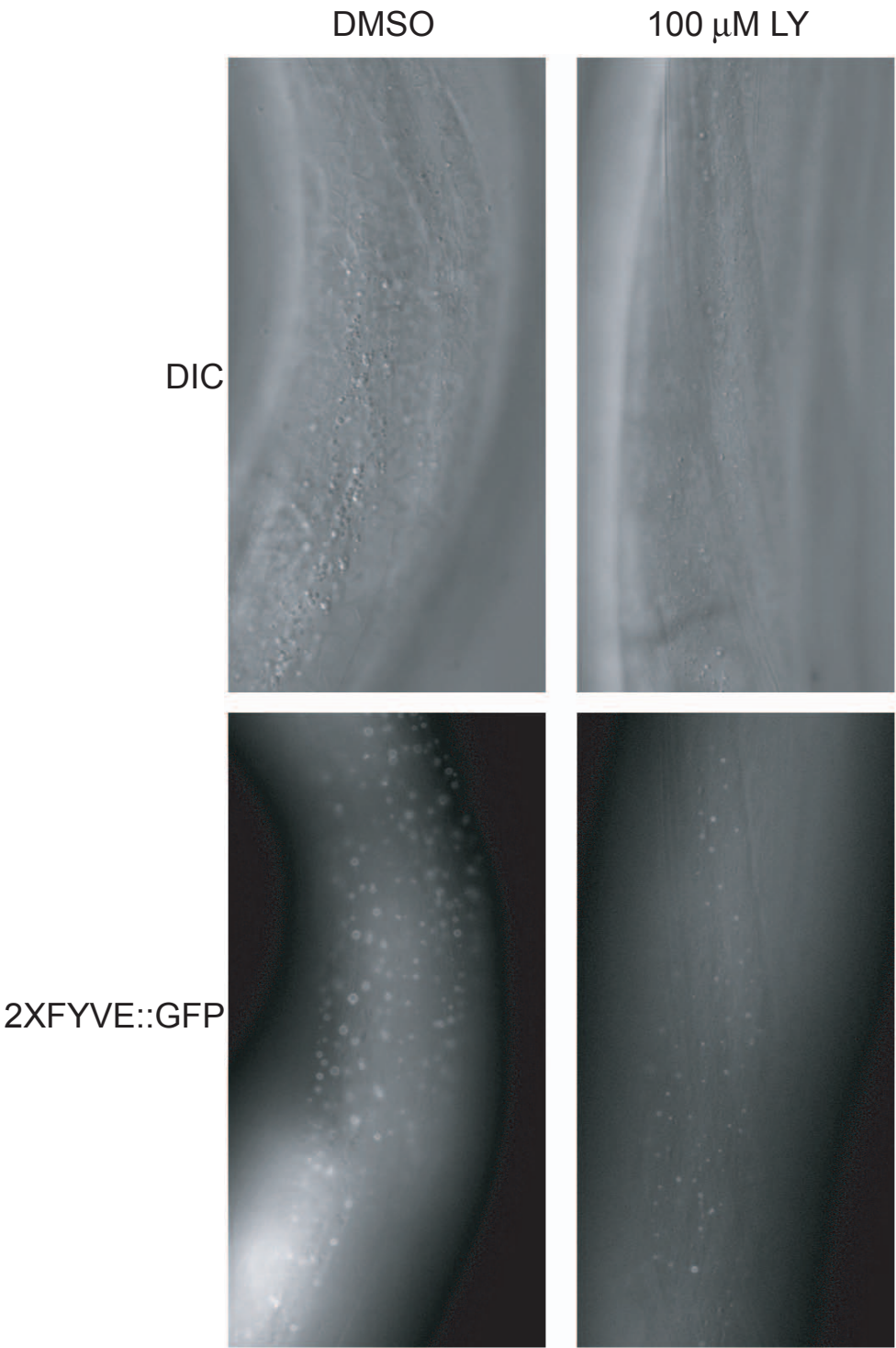
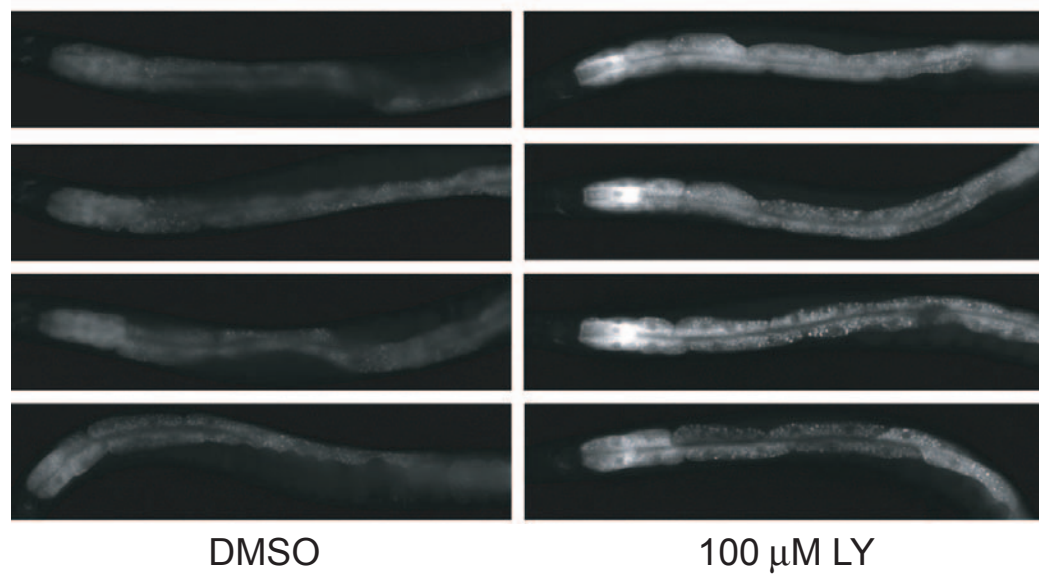


Figure 4-12

A



B

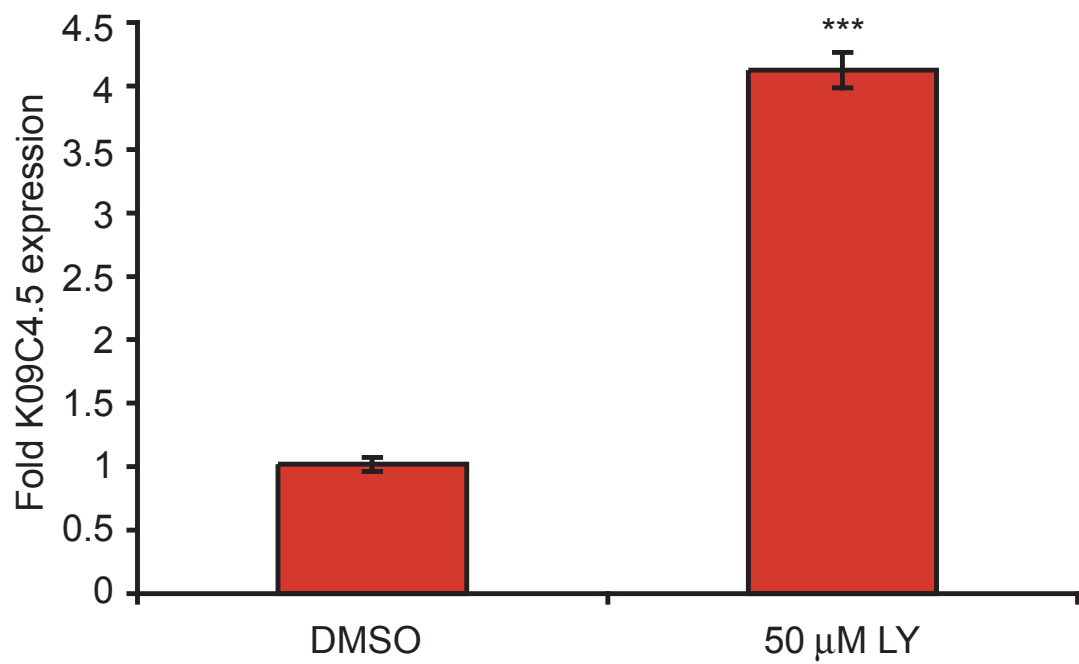
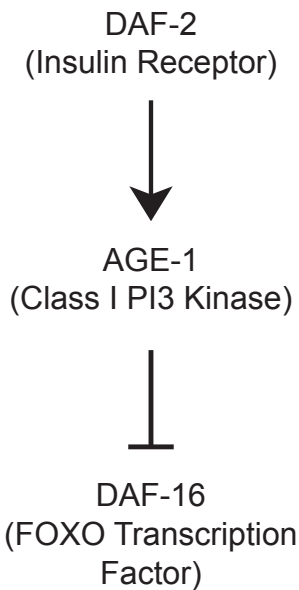


Figure 4-13

A



B

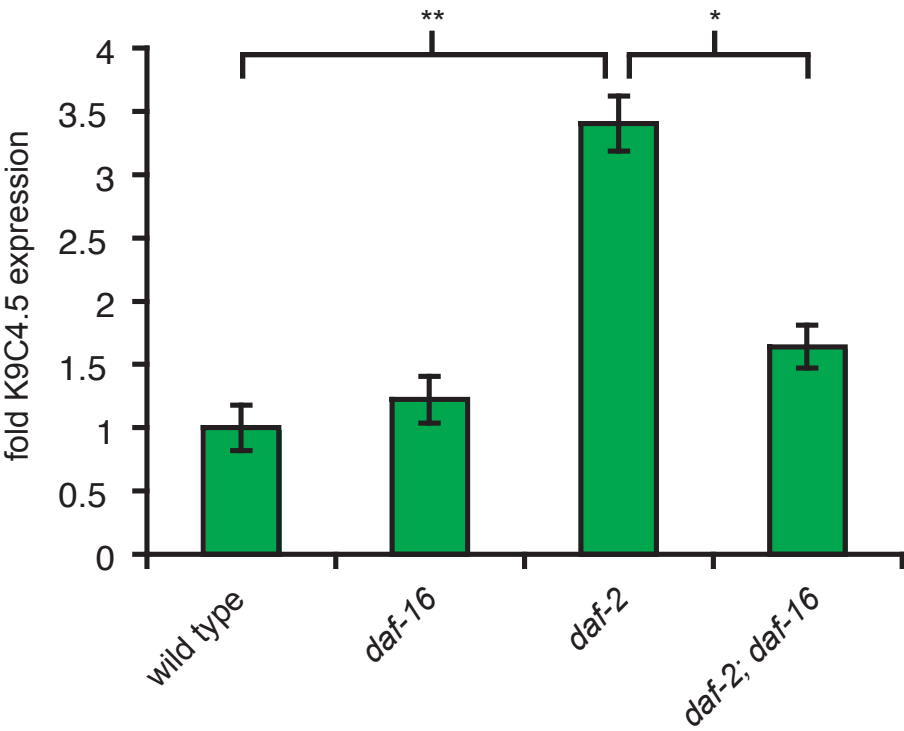


Figure 4-14

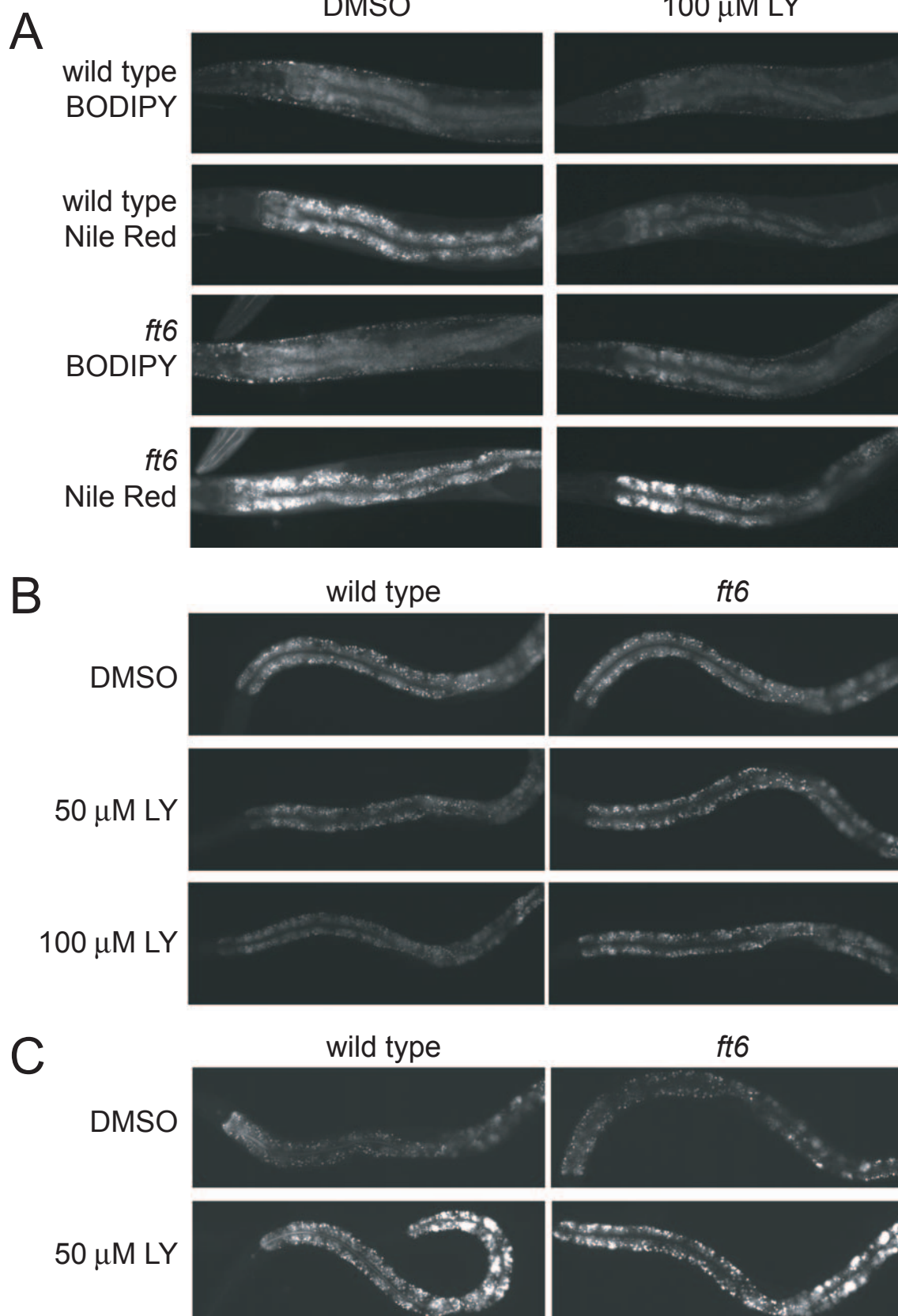
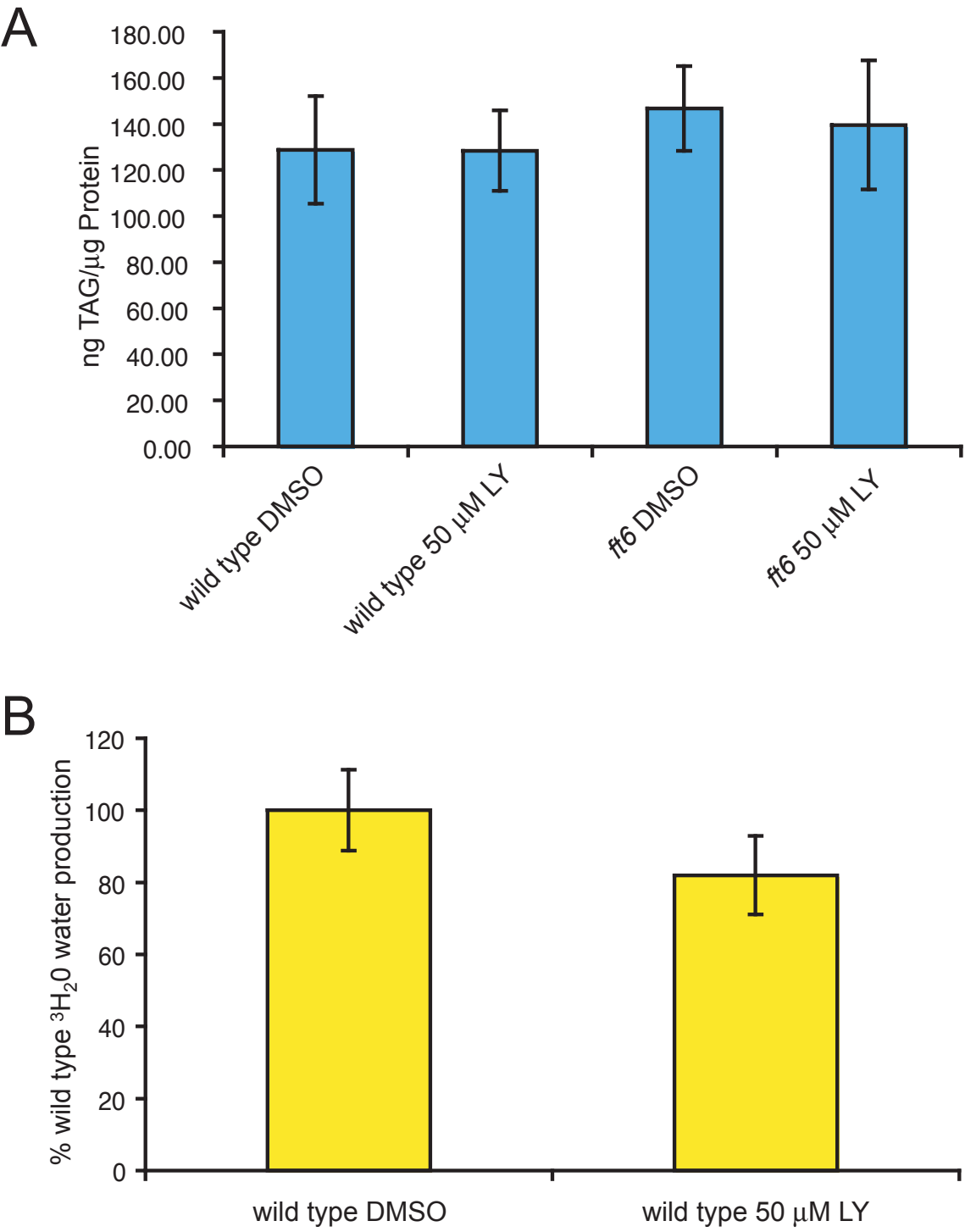


Figure 4-15



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