INTRODUCTION

Cytochromes P450 (CYPs) are mono-oxygenase enzymes that are found in most animals and plants. CYPs are known to oxidize a wide variety of xenogenous compounds and also metabolize endogenous compounds such as steroids and fatty acids. From a toxicological perspective, understanding the endogenous functions of CYPs is important as the induction of these enzymes with xenobiotics is likely to affect the physiological processes involving CYPs (Amacher, 2010). Several studies have shown that human, mouse, or rat CYPs hydroxylate or epoxidize metabolically important fatty acids such as linoleic acid (C18:2n6), α-linolenic acid (C18:3n3), arachidonic acid (C20:4n6), eicosapentaenoic acid (C20:5n3), adrenic acid (C22:4n6), and docosahexaenoic acid (C22:6n3) acid into bioactive compounds (reviewed in Konkel and Schunck, 2010). The nematode Caenorhabditis elegans has 77 CYPs that can be classified into 16 families plus five cyp pseudogenes. The physiological importance of C. elegans CYPs can be predicted in gene knockout or knockdown studies. Knockout or RNA interference of cyp-35A2, -3, -4, or -5 leaves nematodes unable to accumulate fat normally (Ashrafi et al., 2003; Menzel et al., 2007). The CYP-35A family proteins are expressed in the intestine, which is a major location of fat stores (Ashrafi et al., 2003). In addition, C. elegans CYP-29A3 and CYP-33E2 are known to metabolize eicosapentaenoic acid into epoxy and hydroxy derivatives that serve as important endogenous signaling compounds (Kulas et al., 2008). CYP-31A2 and -31A3 are required for the synthesis of lipids that are essential for embryonic development in C. elegans (Benenati et al., 2009). Moreover, C. elegans CYP-22A1 (a.k.a. DAF-9) is known to make steroidal ligands that bind to the nuclear hormone receptor (NHR) DAF-12, and thereby regulate several developmental processes (Gerisch et al., 2001; Jia et al., 2002).

Several factors are involved in the regulation of C. elegans fat stores. Initially, fat is stored as triacylglycerols both in intestinal lysosome-related organelles and in lipid droplets found in intestinal and epidermal skin-like cells and mobilized by lipases (Schroeder et al., 2007; Mullaney and Ashrafi, 2008; Zhang et al., 2010). Fatty acids are used for energy in the process of beta-oxidation, which occurs in peroxisomes and mitochondria and whose end product is acetyl-CoA (Zhang et al., 2010). If beta-oxidation is impaired, the lipid droplets grow in size (Zhang et al., 2010). The nematode takes up fatty acids from the diet, primarily as triglycerides, and also synthesizes them de novo from acetyl-CoA (Watts and Browse, 2002; Rappeleye et al., 2003). C. elegans can make all its necessary unsaturated and long chain fatty acids from short chain fatty acids with desaturase and elongase enzymes, respectively, and does not need to obtain essential fatty acids from diet as humans do.

Background: Cytochrome P450s (CYPs) are mono-oxygenases that metabolize endogenous compounds, such as fatty acids and lipid signaling molecules, and furthermore have a role in metabolism of xenobiotics. In order to investigate the role of CYP genes in fat metabolism at the molecular level, four Caenorhabditis elegans mutants lacking functional CYP-35A1, CYP-35A2, CYP-35A4, and CYP-35A5 were characterized. Relative amounts of fatty acids, as well as endocannabinoids, which regulate weight gain and accumulation of fats in mammals, were measured while fat contents in worms were visualized using Oil-Red-O staining. Results: The cyp-35A1 and cyp-35A5 mutants had a significantly lower intestinal fat content than wild-type animals, whereas cyp-35A2 and cyp-35A4 mutants appeared normal. The overall fatty acid compositions of CYP mutants did not alter dramatically, although modest but significant changes were observed. cyp-35A1 and cyp-35A5 mutants had significantly higher levels of C18:1n7 and lower C18:2n6c. All four mutants had higher relative amounts of C18:1n7 than the wild-type. In the cyp-35A5 mutant, the levels of the endocannabinoid anandamide were found to be 4.6-fold higher than in wild-type. Several fatty acid synthesis genes were over-expressed in cyp-35A1 including fat-2. Feeding oleic or elaidic triglycerides to wild-type animals demonstrated that cyp-35A1 transcriptional levels are insensitive to environmental exposure of these fats, while cyp-35A2, cyp-35A4, and cyp-35A5 were significantly down regulated. Conclusion: These results demonstrate a dynamic role for CYP-35A subfamily members in maintaining the diversity of fatty acid profiles in C. elegans, and more generally highlight the importance of CYPs in generating both structural and signaling fatty acid functions in other organisms.

Keywords: cytochrome P450, fatty acids, metabolism, gene expression, model organism
CYPs can metabolize AEA or its hydrolysis products, which can act as a receptor for AEA (Zygmunt et al., 2001; McPartland et al., 2006).

Fatty acids can be metabolized into various signaling molecules (Konkel and Schunck, 2010). The fatty acid arachidonic acid is the molecular starting point of the arachidonic acid cascade, which includes the production of endocannabinoids. The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are derived from membrane phospholipids through several routes (Awumey et al., 2008; Snider et al., 2010). In mammals, endocannabinoids modulate neurotransmission, affect mood and behavior, and are involved in inflammation. Over activity of the endocannabinoid system has been also associated with obesity by acting through the cannabinoid receptor CB1 in several tissues such as brain, adipose tissue, liver, pancreas, and skeletal muscle (Matias and Di Marzo, 2007; Maccarrone et al., 2010). CB1 antagonists alleviate obesity and improve the plasma lipid profile and insulin sensitivity in obese mice and rats (Cota et al., 2009; Jourdan et al., 2010). Endocannabinoids are also found in C. elegans (Lehtonen et al., 2008) although this nematode lacks direct orthologs of CB1 and CB2 cannabinoid receptors (Elphick and Egertová, 2001; McPartland et al., 2006). C. elegans do have vanilloid receptors which can act as a receptor for AEA (Zygumnt et al., 1999). CYPs can metabolize AEA or its hydrolysis product arachidonic acid (C20:4n6) into signaling molecules such as hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and 2-epoxyeicosatrienoyl glycerols (2-EGs; Bornheim and Egertová, 2001; McPartland et al., 2006). The enzymes responsible for fatty acid synthesis and degradation are expressed in the fat-storing cells and regulated by transcription factors such as NHR-49 (Van Gilst et al., 2008).

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WORM STRAINS AND THEIR GROWTH

The worm strains RB1788, cyp-35A1(ok2306; 1494 bp deletion including first four exons of the coding region; VC710, cyp-35A2(gk317; 1245 bp deletion including four to seven coding exons and 3’UTR); RB1294, cyp-35A4(ok1393; ~600 bp deletion); RB1613, cyp-35A5(ok1985; 367 bp deletion in three to four coding exon); and CB1370, daf-2(e1370, missense 1462 P–S) were received from the Caenorhabditis Genetics Center (Twin Cities, MN, USA). The nematodes were grown in standard conditions (Brenner, 1974).

FAT STAINING

Oil-Red-O (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to stain whole animals. The fatty acid staining was performed as described by O’Rourke et al. (2009). Synchronized day-1 adults were collected from plates and washed three times with 1× PBS. The worms were allowed to settle by gravity. To permeabilize the cuticle, 120 μL of PBS and 120 μL of 2× MRWB buffer (Modified Ruvkun’s Witches Brew; 160 mM KCl, 40 mM NaCl, 20 mM NaEGTA, 10 mM Spermidine HCl, 30 mM Na-PIPES pH 7.4, 50% methanol) containing 2% paraformaldehyde was added. The 2× MRWB buffer used in our experiments contained an equal concentration of EDTA in place of NaEGTA, and an equal concentration of HEPES in place of Na-PIPES. The nematodes were incubated at room temperature for 1 h with gently shaking and then washed with 1× PBS. To dehydrate the worms, 300 μL of 60% isopropanol was added and the samples were incubated for 15 min. The isopropanol solution was removed and 1 mL of 60% Oil-Red-O was added, as prepared in O’Rourke et al. (2009). The samples were incubated overnight with gentle shaking. The dye was removed and the nematodes were washed twice with 1× PBS, containing 0.01% Tween. The worms were resuspended in 1× PBS with 0.01% Tween, pipetted onto agarose pads and imaged with an Olympus AX70 fluorescence microscope (Olympus, Tokyo, Japan) with a UPlan Apo 60X/0.90 objective.

OVERALL FATTY ACID COMPOSITION

Fatty acid profiles were measured from CYP mutants and N2 strains that were synchronized to the L4 stage by bleaching. A method similar to Watts and Browse (2000, 2002) was used for sample analysis. Briefly, to a 10-μL sample of worm pellet collected by centrifugation (~350 g), 0.5 mL of 2.5% H2SO4 in methanol was added and samples were heated at 60°C for 1 h. After cooling the samples to room temperature, 1 mL of hexane
and 1.5 mL of H2O were added and the mixture was vortexed. The phases were separated and the organic phase was evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 100 μL of hexane for analysis. Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography coupled to electron ionization mass spectrometry (Agilent Technologies, Palo Alto, CA, USA) using a bis-cyanopropyl capillary column (100 m x 0.25 mm i.d.) with 0.20 μm film thickness (SP-2560, Supelco, Bellefonte, PA, USA) for analysis with pulsed split injection (1 μL). The oven temperature was held at 180°C for 2 min, then increased to 240°C at 3°C/min rate and held at 240°C for 8 min. The carrier gas was helium with constant flow of 21 cm/s. The temperatures of the MS transfer line heater, ionization source, and quadrupole were maintained at 250, 230, and 150°C, respectively. In the GC/EI–MS full scan and the selected ion monitoring modes were used for qualitative and quantitative analysis of FAMES, respectively.

**GENE EXPRESSION OF FATTY ACID SYNTHESIS AND BETAL-OXIDATION GENES**

Total RNA was extracted from four separate L4 larval stage C. elegans cultures with RiboPure™ Kit (Ambion, Austin, TX, USA). For each replicate, 0.5 μg of total RNA was reverse transcribed in a volume of 19 μL using 2 μL dNTP Mix (10 mM each, Fermentas Life Sciences, Vilnius, Lithuania), 4 μL 5X Reaction Buffer, and 1 μL RevertAid Minus M-MuLV Reverse Transcriptase (200 u/μL; Fermentas Life Sciences). For each replicate, 0.5 μg of total RNA was reverse transcribed in 50 μL consisting of 12.5 μL of Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences), 0.2 μM (final concentration) of forward and reverse primers each in nuclease free H2O and 2.5 μL of the diluted RT reaction. The PCR reactions were performed in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Life Sciences, Hercules, CA, USA). The oligo primers were purchased from Oligomer (Helsinki, Finland) and sequences were: act-1 forward, 5′-TGG GTA TGG GAC AGA AGG AC-3′; act-1 reverse; 5′-CAT CCC AGT TGG TGA CGA TA-3′; fat-1 forward, 5′-CGC CTT CTC ACC ACT CTG CT-3′; fat-1 reverse, 5′-TCC ACA CGT GTC CAT GT-3′; fat-2 forward, 5′-TCC CGG CTC TTC-3′; fat-2 reverse, 5′-GCC CAG AGA CGC AAT ATC-3′; fat-4 forward, 5′-ACA TCC AGG TGG TAG TGC AA-3′; fat-4 reverse, 5′-CTG GTA TCT GTG CTT GTG TG-3′; fat-5 forward, 5′-CGC TCA TAT GGG ATG GTT TG-3′; fat-5 reverse, 5′-CTGC CGG AGA GCA AAA G-3′; fat-6 forward, 5′-GCC AGA GCC CCT TTA TTA TG-3′; fat-6 reverse, 5′-TTG AAG AGC GCC GG CTT TGA GGT TCT-3′; fat-7 forward, 5′-ACA TCC AGG TGG TAG TGC AA-3′; fat-7 reverse, 5′-GCA GCC ATT GGA CCT TAC GA-3′; fat-9 forward, 5′-GAG GAG GCC TCT CAG TCG TA-3′; fat-9 reverse, 5′-GAA TTA AAA G-3′; cis-triolein, above 75°C for the trans-triolein), each oil was dispersed into hot agar by magnetic stirring drops of oil of a previously weighted volume, to achieve a final concentration of 25 μM in the agar. Plates were cooled quickly after pouring the agar, and then stored in a cool, dark place before use, in order to keep these triglycerides evenly distributed throughout the solidified agar. Higher concentrations of triglycerides eventually resulted in the appearance of oil droplets on the agar surface. Gravid worms grown on NGM agar were collected and embryos obtained by bleaching. Embryos were then grown on triglyceride plates and collected for analyses at the L4 stage (~48–56 h later).

Gene expressions of the Cyp-35A1, Cyp-35A2, Cyp-35A4, and Cyp-35A5 genes in wild-type nematodes with and without feeding of fatty acids were measured as described in Section “Gene Expression of Fatty Acid Synthesis and Beta-Oxidation Genes.” The oligo primers were purchased from Oligomer (Helsinki, Finland). Their sequences were: cyp-35A1 forward, 5′-CGG CAG AAG CCG TTA AAA G-3′; cyp-35A1 reverse, 5′-TGA CCA GTC ATC CAC AAA TCG-3′; cyp-35A2 forward, 5′-TTC TCC CTT CAA GCA TTT AGG A-3′; cyp-35A2 reverse, 5′-ATC GAA AAA TTC AGA GCC GTC ATG T-3′; cyp-35A4 forward, 5′-ACC AAA TCA AGT CGT GGA GGT A-3′; cyp-35A4 reverse, 5′-GCC TGT TAT CCA TAA ATC ACC AA-3′; cyp-35A5 forward, 5′-TAC CTT TGG ACA ACT GGT GAT-3′; cyp-35A5 reverse, 5′-AAG TCT CAT AAT CGG CAA TGC T-3′.

**ENDOCANNABINOIDs**

The endocannabinoids were measured with liquid chromatography coupled to an electrospray-ionization triple quadrupole mass spectrometer (ESI–LC/MS/MS; Lehtonen et al., 2008). Briefly, synchronized fourth larval stage (L4) nematodes were collected from plates with M9 buffer. Twenty microliters of vortexed sample was transferred to an Eppendorf tube. One milliliter of MeOH was added and the samples were homogenized with a Soniprep 150 homogenizer (MSE Ultrasonic Disintegrator, MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England). Lipids were extracted by adding 2 mL of CHCl3 and 1 mL of H2O, followed by centrifugation at 1500 × g and incubation at 10°C for 10 min to separate the phases. The aqueous upper layer was removed and the organic layer transferred to a screw-capped test tube. The lipid extraction was repeated on the obtained sample. The sample was evaporated to dryness under N2 and the residue was reconstituted in 50 μL of ice-cold ACN. The residue was allowed to dissolve for 5 min, after which 20 μL of H2O was added. The sample was centrifuged at 12000 × g and 10°C for 10 min and transferred to an HPLC sample vial.

**EXPRESSION OF cyp-35A GENES IN RESPONSE TO FEEDING OF FATTY ACIDS**

The troilein- and trioilaidin-glycerides were purchased from Nu-Chek Prep (Elysian, MN, USA). Troilein-glyceride contained the pure cis-18:1n9 fatty acid. Trioilaidin contained the pure trans-18:1n9 fatty acid. After melting the triglyceride in a water bath (above 25°C for the cis-troilein, above 75°C for the trans-trioilaidin), each oil was dispersed into hot agar by magnetic stirring drops of oil of a previously weighted volume, to achieve a final concentration of 25 μM in the agar. Plates were cooled quickly after pouring the agar, and then stored in a cool, dark place before use, in order to keep these triglycerides evenly distributed throughout the solidified agar. Higher concentrations of triglycerides eventually resulted in the appearance of oil droplets on the agar surface. Gravid worms grown on NGM agar were collected and embryos obtained by bleaching. Embryos were then grown on triglyceride plates and collected for analyses at the L4 stage (~48–56 h later).
Anandamide and 2-AG were quantified by liquid chromatography (Agilent 1200 series rapid resolution LC system, Agilent Technologies, D-Waldbronn) coupled with an ESI triple quadrupole mass spectrometer (Agilent 6410 triple quadrupole LC/MS, Agilent Technologies, Palo Alto, CA, USA). Ten microliters of sample solution was injected onto a reversed phase HPLC column (Zorbax Eclipse XDB-C18 rapid resolution HT 2.1 mm × 50 mm, 1.8 mm, Agilent Technologies, Palo Alto, CA, USA) using an isocratic mobile phase consisting of 55% of 0.1% HCOOH in ACN and 45% of 0.1% HCOOH in H₂O, delivered at 200 μL/min. Column temperature was maintained at 40°C and the autosampler tray temperature was set at 10°C. The following ionization conditions were used: ESI, positive-ion mode; drying gas (N₂) temperature, 300°C; drying gas flow, 10 L/min; nebulizer pressure, 50 psi, and cap. voltage, 4000 V. Detection was performed using multiple reaction monitoring (MRM) with the following transitions: m/z 348 → 62 for AEA, m/z 356 → 63 for AEA-d₈, m/z 379 → 287 for 2-AG, and 387 → 294 for 2-AG-d₈. Fragmentor voltage and collision energy for AEA, AEA-d₈, 2-AG, and 2-AG-d₈ were 120 and 10 V, 120 and 12 V, 130 and 8 V, and 125 and 10 V, respectively. Dwell time was 100 ms for each transition, and mass resolution for MS1 and MS2 quadrupoles were 2.4 and 1.2 FWHM, respectively, for AEA, and 0.7 and 0.7 FWHM, respectively, for AEA-d₈, 2-AG, and 2-AG-d₈. Deuterated internal standards, AEA-d₈ and 2-AG-d₈, were used for quantification.

RESULTS

PHYLOGENETICS

The C. elegans CYP-35 family is most closely related to C. elegans CYP-34 family. The closest human CYP families to C. elegans CYP-35 are CYP-1 (Hs1) and CYP-2 (Hs2). However, members of the major human families CYP-1 and CYP-2 clearly share more similarities with each other than with C. elegans families. Human CYP-3 (Hs3) and CYP-4 (Hs4) families are more closely related to C. elegans families other than CYP-35 (Figure 1).

Within the C. elegans CYP-35A subfamily the peptides CYP-35A2 and -3 are the most similar (Figure 1). CYP-35A1 is the most different. Subfamilies B, C, and D are not as expanded as subfamily A. The amino acid sequences of the CYP-35A proteins are very similar at the N terminus from amino acid 0 to 100, and also from amino acid 300 to 400 with minor amino acid differences. CYP-35A1 is missing a 38 amino acid sequence that the other CYP-35A proteins have in the middle of the peptide chain (amino acids 254–291), and it has additional amino acids closer to the C terminus (Figure S1 in Supplementary material).

FAT STAINING IN cyp-35A MUTANTS

Wild-type N2 strain animals feeding on bacteria show abundant Oil-Red-O staining, particularly in the intestine and mature oocytes (Figures 2A–C). Animals starved for a day show loss of staining, in the intestine and less in oocytes (Figures 2D–F). The daf-2 mutant, as a positive control, showed heavy fat staining in the intestine (Figures 2G–I). In contrast, the cyp-35A1 mutant showed light Oil-Red-O staining in the intestine, mainly in the anterior part, and oocytes (Figures 2J–L), resembling the starved wild-type that also had staining in the eggs but not visible amounts in the intestine. The cyp-35A2 and cyp-35A4 mutants had about the same level of Oil-Red-O staining as the wild-type (Figures 2M–R). The cyp-35A5 had some Oil-Red-O staining in the intestine but visibly less than wild-type (Figures 2S–U).

FATTY ACID COMPOSITION AND ENDOCANNABINOID CHANGES IN cyp-35A MUTANTS

The overall fatty acid compositions of CYP mutants did not alter dramatically, although modest but significant changes were observed (Figure 3). Compared to wild-type animals, cyp-35A1

CYP-35A genes in C. elegans

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mutants had significantly higher levels of C18:1n7 and lower C18:2n6c. All four mutants had higher relative amounts of C18:1n7 than the wild-type (Figure 3).

mutants had significantly higher C16:0, C18:1n7, C18:3n6, and C20:4n6, while having significantly lower levels of C18:2n6c, C20:4n3, and C20:5n3 fatty acids. In concordance, cyp-35A5 mutants had significantly higher levels of C18:1n7 and lower C18:2n6c. All four mutants had higher relative amounts of C18:1n7 than the wild-type (Figure 3).
The endocannabinoid AEA was found to be 4.6-fold higher in the cyp-35A5 mutant than in the wild-type (Figure 4). 2-AG levels were not significantly altered in any of the mutants.

**GENE EXPRESSION CHANGES OF FATTY ACID SYNTHESIS GENES AND acs-2**

In the cyp-35A1(ok2306) mutant many genes required in the synthesis of long chain fatty acids appeared to be expressed in 2–3.5 times higher amounts than in wild-type (Figure 5A). The increases in fat-2, fat-3, fat-5, fat-6, fat-7, elo-1, and elo-6 were significant ($p < 0.05$). In a different cyp-35A1(ok1414) mutant allele strain, only fat-2 was significantly over-expressed, but all the measured genes were found in somewhat higher amounts than in the wild-type (data not shown).

In the cyp-35A5 mutant many fatty acid synthesis genes seemed to be under-expressed, although only the changes in fat-1 and fat-3 were significant. The fatty acid beta-oxidation gene acs-2 was slightly (1.7-fold) over-expressed in the cyp-35A2 mutant and under-expressed (fold change 0.8) in the cyp-35A5 mutant (data not shown).

A map of the fatty acid metabolism pathway was then drawn and significant changes in gene expression and fatty acids were placed onto the map. The top part of the map involving C16:0, C16:1, C18:0, C18:1n7 appears to be concordant: gene expression increases in fat-5 for the cyp-35A1 mutant are in agreement with increases in C18:1n7. The middle portion of the map including C18:1n9, C18:2n6, C18:3n3, C18:3n6, C18:4n3 does not appear to be concordant. The bottom part of the map including C20:3n6, C20:4n6, and C20:5n3 appears concordant, particularly the increases in C20:4n6 due to increases in elo-1 expression in cyp-35A1 animals (Figure 5B).

**EXPRESSION OF cyp-35A SUBFAMILY GENES IN RESPONSE TO FEEDING OF cis- AND trans-C18:1n9**

In order to determine whether or not cyp-35A family genes respond to fat feeding, we fed wild-type animals oleic or elaidic triglycerides, which are the cis- and trans-triglycerides of C18:1n9, respectively. In wild-type *C. elegans*, the cyp-35A1 gene was not responsive to fat feeding, while cyp-35A2, cyp-35A4, and cyp-35A5 significantly decreased expression (Table 1). There was no differentiation when animals were fed cis- or trans-isomers of these triglycerides (Table 1).

**DISCUSSION**

**PHYLOGENETICS**

*Caenorhabditis elegans* CYP peptide sequences clustered similarly as in an early phylogenetic study by Gotoh (1998). The relatively large number of paralogs in the cyp-35 family indicates that these
CYP-1 and CYP-2, suggesting their relatively recent evolution probably in response to xenobiotic challenges. Those families were clustered together with several *C. elegans* CYP families (such as *cyp-35* family), similarly as in fish and insects (Nelson, 2003; Feyereisen, 2006), indicating an evolutionally old distinction between the major CYP clades, probably due to their early separated endogenous functions, which in human include metabolism of fatty acid derivatives (Arnold et al., 2010). In the genome, the *cyp-35* genes are located in a cluster on chromosome V, adjacent to each other and *cyp-34* family genes. CYPs whose genes appear in clusters are more likely to be involved in xenobiotic metabolism than CYPs whose genes reside isolated from other *cyp* genes in the genome (Thomas, 2007).

The closest human counterparts of the CYP-35A family, the highly diverse families CYP-1 and CYP-2, suggesting their relatively recent evolution probably in response to xenobiotic challenges. Those families were clustered together with several *C. elegans* CYP families (such as *cyp-35* family), similarly as in fish and insects (Nelson, 2003; Feyereisen, 2006), indicating an evolutionally old distinction between the major CYP clades, probably due to their early separated endogenous functions, which in human include metabolism of fatty acid derivatives (Arnold et al., 2010). In the genome, the *cyp-35* genes are located in a cluster on chromosome V, adjacent to each other and *cyp-34* family genes. CYPs whose genes appear in clusters are more likely to be involved in xenobiotic metabolism than CYPs whose genes reside isolated from other *cyp* genes in the genome (Thomas, 2007). The closest human counterparts of the CYP-35A family, the highly diverse families CYP-1 and CYP-2, metabolize both exogenous and endogenous compounds, the latter including omega-3 and -6 fatty acids and their derivatives (Schwarz et al., 2004; Fer et al., 2008; enzymes may function at least partly in xenobiotic metabolism (Thomas, 2007). This is also supported by studies showing that the *cyp-35A* family genes are induced by a number of xenobiotics (Menzel et al., 2001, 2005; Reichert and Menzel, 2005; Roh et al., 2007; Chakrapani et al., 2008). Further, as shown in phylogenetic comparisons between fish, insect, and human CYPs (Nelson, 2003; Feyereisen, 2006), we observed high similarities with human families

### Table 1 | Fold change ± SEM of the expression of *cyp-35A* family genes.

|          | *cyp-35A1* | *cyp-35A2* | *cyp-35A4* | *cyp-35A5* |
|----------|------------|------------|------------|------------|
| N2 cis/no feeding | 0.93 ± 0.19 | 0.66 ± 0.05* | 0.53 ± 0.05*** | 0.63 ± 0.05* |
| N2 trans/no feeding | 1.12 ± 0.61 | 0.38 ± 0.05** | 0.56 ± 0.10* | 0.50 ± 0.09* |

Significances of the alterations were determined with Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

![Figure 5](link_to_image) | **(A)** Changes in the expression levels of fatty acid synthesis genes (fold change compared to wild-type ± SEM). *t*-test *p*-value < 0.05, **t*-test *p*-value < 0.01, ***t*-test *p*-value < 0.001. **(B)** Schema of the synthesis of fatty acids and the enzymes involved. ▲ = fatty acid percentage or gene expression increased in *cyp-35A1* mutant; ▼ = fatty acid percentage or gene expression decreased in *cyp-35A1* mutant; ▲ = fatty acid percentage or gene expression increased in *cyp-35A5* mutant; ▼ = fatty acid percentage or gene expression decreased in *cyp-35A5* mutant.
Arnold et al., 2010). Nematodes C. briggsae and C. remanei have CYP-35A subfamilies but they are not as expanded as the C. elegans CYP-35A subfamily (data not shown). Vertebrates or insects do not have a CYP-35 family suggesting that CYP-35 enzymes may have a nematode-specific endogenous function.

cyp-35A1 AND cyp-35A5 MUTANTS HAVE A DEPLETED FAT CONTENT AS VISUALIZED BY OIL-RED-O STAINING
The lack of functional CYP-35A1 and CYP-35A5 leads to decreases in intestinal fat (Figure 2). This suggests that these CYPs are necessary, but not sufficient to maintain fat storage. The molecular mechanism by which these CYPs act may be complex. Genome-wide RNAi studies in C. elegans have revealed 305 gene inactivations that reduce body fat (Ashrabi et al., 2003). In addition to CYPs, signal transduction enzymes, receptors, transporters, and energy metabolism enzymes were found. Among the best studied fat storage regulators in C. elegans is NHR-49, a NHR, which has been shown to influence the regulation of 13 genes involved in energy metabolism (Van Gilst et al., 2005a,b). Several of these (fat-5, fat-6, and fat-7) overlap with those found to be regulated in this study, which suggests that CYPs and NRHs may share common pathways in the regulation of fat storage.

In earlier fat staining experiments with Nile Red, the cyp-35A2(gk317), cyp-35A3, cyp-35A4(ok1393), and cyp-35A5(ok1985) mutants were deficient in fat accumulation, but the cyp-35A1(ok1414) mutant was normal (Menzel et al., 2007). The study of Menzel et al. (2007) used a different allele of cyp-35A1 compared to our study, but the other strains used were the same. The reason for the different results could be the stains used. The Nile Red dye stains the lysosome-related organelles but not the lipid droplets (O’Rourke et al., 2009), and these differences could explain our results, as we used Oil-Red-O. Recently, other laboratories have used Oil-Red-O in place of Nile Red. In support of this hypothesis, cyp-35A5 mutants respond homeostatically to lower fats by increasing endocannabinoids, why do cyp-35A1 mutants not respond in the same manner? First, lack of the CYP-35A1 protein appears to result in lower overall fat (Figure 2) and increases in both fatty acid desaturation and elongation gene expressions (Figure 5). These appear to suggest a role for CYP-35A1 in maintaining constitutive fat levels. In support of this hypothesis, cyp-35A1 gene expression does not respond to fat feeding while other cyp-35A1 family members do (Table 1). Moreover, endocannabinoid levels appear normal in cyp-35A1 mutants.

CONCLUSION
Taken together, these results show that the CYP-35A subfamily plays a part in the dynamic regulation of the variety of different fatty acid molecules in C. elegans. The lack of CYP-35A1 and CYP-35A5 decreases intestinal fat stores. However, the mechanism by which this is achieved may differ, as the transcriptional levels of fatty acid metabolism genes differ dramatically between these two mutants. Moreover, CYP-35A1 does not appear to respond transcriptionally to fat ingestion, whereas CYP-35A5 and other CYP-35 members consistently decrease gene expression. The lack of CYP-35A5 affects AEA levels. Taken with the modest changes in fatty acid profiles, these results suggest a stronger role for CYP-35A members in regulating fatty acid derived signaling molecules, rather than structural fatty acids, in C. elegans. In summary, these results demonstrate an important role for CYP-35A members in regulating fat metabolism and signaling molecules in C. elegans. These results thus provide a framework for future mechanistic studies...
to investigate fatty acid signaling in pharmacology or toxicology contexts using *C. elegans*. Finally, these findings provide a basis to predict toxicity in higher organisms including humans.

**Authorization for the use of experimental animals:** Use of genetically modified organisms (GMO) in this study was approved by the Finnish GMO authority (Reg. number 2/E/09).

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online [http://www.frontiersin.org/Predictive_Toxicity/10.3389/fphar.2011.00012/abstract](http://www.frontiersin.org/Predictive_Toxicity/10.3389/fphar.2011.00012/abstract)

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