Eicosanoid formation by a cytochrome P450 isoform expressed in the pharynx of Caenorhabditis elegans

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Caenorhabditis elegans harbours several CYP (cytochrome P450) genes that are homologous with mammalian CYP isoforms important to the production of physiologically active AA (arachidonic acid) metabolites. We tested the hypothesis that mammals and C. elegans may share similar basic mechanisms of CYP-dependent eicosanoid formation and action. We focused on CYP33E2, an isoform related to the human AA-epoxygenases CYP2C8 and CYP2J2. Co-expression of CYP33E2 with the human NADPH–CYP reductase in insect cells resulted in the reconstitution of an active microsomal mono-oxygenase system that metabolized EPA (eicosapentaenoic acid) and, with lower activity, also AA to specific sets of regioisomeric epoxy- and hydroxy-derivatives. The main products included 17,18-epoxyeicosatetraenoic acid from EPA and 19-hydroxyeicosatetraenoic acid from AA. Using nematode worms carrying a pCYP33E2::GFP reporter construct, we found that CYP33E2 is exclusively expressed in the pharynx, where it is predominantly localized in the marginal cells. RNAi (RNA interference)-mediated CYP33E2 expression silencing as well as treatments with inhibitors of mammalian AA-metabolizing CYP enzymes, significantly reduced the pharyngeal pumping frequency of adult C. elegans. These results demonstrate that EPA and AA are efficient CYP33E2 substrates and suggest that CYP–eicosanoids, influencing in mammals the contractility of cardiomyocytes and vascular smooth muscle cells, may function in C. elegans as regulators of the pharyngeal pumping activity.

Key words: arachidonic acid, Caenorhabditis elegans, cytochrome P450, eicosanoid, eicosapentaenoic acid, long-chain polyunsaturated fatty acid.

INTRODUCTION

The n−6 and n−3 LC-PUFAs (long-chain polyunsaturated fatty acids), such as AA (arachidonic acid) (C_{20:4}ω-6) and EPA (eicosapentaenoic acid) (C_{20:5}ω-3), are essential for health and development of animals. Whereas n−6 and n−3 LC-PUFAs are accessible to mammals only via nutrition, the nematode Caenorhabditis elegans has the capacity to synthesize both classes of these important fatty acids de novo [1,2]. Moreover, unlike plants, de novo LC-PUFA synthesis in C. elegans does not end at the level of C_{18} fatty acids, but proceeds further to yield AA and EPA as the main products. This makes C. elegans an attractive model to analyse the multiple physiological functions of LC-PUFAs. Loss-of-function mutations in LC-PUFA biosynthesis genes result in pleiotropic defects, including mechanosensory, osmosensory and olfactory deficits [3]. Nematode worms lacking a functional Δ^8 desaturase (encoded by the fat-3 gene) are unable to synthesize any fatty acid beyond linoleic (C_{18:2}ω-6) and linolenic acid (C_{18:3}ω-3), leading to reduced brood size and neuromuscular defects [1,2,4]. fat-3(wa22) mutants also show reduced pharyngeal pumping activity, suggesting that AA and EPA are involved in the regulation of pharynx activity [4]. Nearly all of these deficits can be rescued by feeding the mutant nematodes with the missing LC-PUFAs [4]. Moreover, LC-PUFAs are necessary for signalling in oocytes during recruitment of sperm to the spermatheca [5]. These data indicate important roles for LC-PUFAs in various behavioural and developmental capacities of C. elegans. However, the molecular mechanisms of how LC-PUFAs contribute to these fundamental processes are largely unclear.

In general, LC-PUFA actions may include direct effects on membrane structures, ion channels and transcription factors. Moreover, LC-PUFAs may serve as precursors of eicosanoids and other signalling molecules. In mammals, eicosanoid biosynthesis proceeds via three pathways that are initiated by COXs (cyclo-oxygenases), LOXs (lipoxygenases) and CYP (cytochrome P450) enzymes [6–10]. Interestingly, C. elegans does not contain any obvious orthologues of mammalian COX and LOX enzymes or of prostanoid and leukotriene receptors [11,12]. However, the C. elegans genome harbours 75 potentially functional CYP genes compared with 57 in humans and 102 in mice [13]. In particular, members of the C. elegans CYP families CYP29 and CYP33 show significant amino acid sequence homologies with mammalian CYP4A4/F and CYP2C2J isoforms that function as AA-hydroxylases and AA-epoxygenases respectively [14]. We demonstrated that epoxy- and...

Abbreviations used: AA, arachidonic acid; CID, collision-induced dissociation; COX, cyclo-oxygenase; CP, chiral-phase; CYP, cytochrome P450; DHET, dihydroxyeicosatetraenoic acid; DTT, dithiothreitol; EEQ, epoxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EPA, eicosapentaenoic acid; GFP, green fluorescent protein; hCPR, human NADPH–CYP reductase; HEET, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LA, dodecanoic (‘lauric’) acid; LC, liquid chromatography; LC-PUFA, long-chain polyunsaturated fatty acid; LOX, lipoxygenase; NGM, nematode growth medium; NP, normal-phase; 17-ODYA, 17-octadecynoic acid; OH-LA, hydroxydodecanoic (‘hydroxylauric’) acid; PPOH, 6-[(2-propargyloxophenyl)hexanoic acid; qRT, quantitative real-time; RNAi, RNA interference; RP, reversed-phase; R_t, retention time.

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hydroxyl metabolites of AA and EPA are endogenous constituents of *C. elegans* [14]. Moreover, we found that these metabolites are produced by a microsomal mono-oxygenase system that can be blocked by typical inhibitors of mammalian CYP-dependent AA metabolism such as 17-ODYA (17-octadecynoic acid) and PPOH [6-(2-propargyloxyphenyl)hexanoic acid]. Our gene-silencing experiments revealed the *emb-8* [15] gene encoded NADPH-CYP reductase [16] as the essential electron-transfer component and identified CYP29A3 and CYP33E2 as the most likely candidates for catalysing the metabolism of AA and EPA in *C. elegans* [14]. Extending the parallels between *C. elegans* and mammals, the nematode also expresses soluble epoxide hydrolases that metabolize CYP-dependent epoxy-metabolites of diverse LC-PUFAs [17].

On the basis of these findings, we hypothesized that *C. elegans* and mammals may share common mechanisms of CYP eicosanoid biosynthesis and action. This would provide the opportunity to use *C. elegans* as a model organism to facilitate the identification of the primary targets of CYP eicosanoids and the elucidation of signalling pathways mediating their physiological functions. As a first step to address these questions, we have focused on CYP33E2 and analysed the substrate specificity and cellular localization as well as the potential role for this CYP isoform in the regulation of the pharyngeal activity in *C. elegans*.

**EXPERIMENTAL**

**Nematode strain and cultivation condition**

The *C. elegans* wild-type strain Bristol N2 and the mutants *fat-1*(wa9) and *fat-3*(wa22) were used throughout the present study. The nematodes were grown at 24°C on NGM (nematode growth medium) agar plates inoculated with *Escherichia coli* OP50 as a food source [18] and were incubated under similar conditions as described previously [14].

**Amplification and cloning of the CYP33E2 cDNA**

Reverse transcription of 4 μg of total RNA was performed at 50°C for 30 min using the Transcriptor High Fidelity cDNA Synthesis Sample Kit (Roche). Subsequently, PCR amplification of the CYP33E2 cDNA was performed using the Phusion® High Fidelity DNA Polymerase (Finnzymes) and the following primer pair: FP-33E2, 5′-CTCTGTTAGAATGATATTATTTATTG-3′, and RP-33E2, 5′-GGTTAAAAACACAAACATTTG-3′. After Taq polymerase-mediated generation of 3′-adenosine overhangs, the PCR product was cloned into the pCR® 2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen).

**Generation of recombinant baculoviruses**

The CYP33E2 cDNA was recloned from pCR® 2.1-TOPO into the pFastBac™1 vector (Invitrogen) using the restriction enzymes HindIII and NotI. The identity of CYP33E2 cDNA was confirmed by full-length sequencing of both DNA strands, performed by LGC Genomics. Recombinant baculoviruses containing the CYP33E2 cDNA under control of the strong polyhedrin promoter were produced using the Bac-to-Bac® Baculovirus Expression System from Invitrogen. After reamplification in Sf9 (*Spodoptera frugiperda*) cells, a virus titre of approximately (1–3) × 10⁸ was achieved.

**CYP33E2 expression in insect cells and preparation of enzymatically active microsomes**

Sf9 cells were grown in ExCell 420 medium (Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. After reaching a cell density of 1.8 × 10⁸ cells/ml, the cultures were co-infected with the recombinant baculoviruses prepared for the expression of CYP33E2 and of the hCPR (human NADPH–CYP reductase), essentially as described previously for the generation of other CYP–hCPR mono-oxygenase systems [19].

In control experiments, the cultures were infected with an empty baculovirus or with either the CYP33E2– or hCPR–baculovirus alone. At 24 h after infection, the medium was supplemented with 5 μM haemin chloride and 100 μM riboflavin. The cells were harvested after a further 24 h, resuspended in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, 0.5 mM DTT (dithiothreitol), 1 μM FAD and 1 μM FMN. The harvested cells were lysed by brief sonication. Microsomes were prepared at 4°C by differential centrifugation (5 min at 3000g, 10 min at 10000 g and 65 min at 100000 g). The microsomes were resuspended and homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA and 0.5 mM DTT, shock-frozen in liquid nitrogen and stored in 30 μl aliquots at −80°C.

The microsomal protein concentration was measured using the method of Lowry et al. [20]. The CYP content was determined by CO difference spectra using a difference absorption coefficient of 91 mM⁻¹·cm⁻¹ for the wavelength pair 450 nm minus 490 nm [21]. The hCPR content was estimated as NADPH–cytochrome c reductase activity using a difference absorption coefficient at 550 nm of 21 M⁻¹·cm⁻¹ [22]. To calculate the reductase content, it was assumed that 4.5 units (μmol of cytochrome c/min) correspond to 1 nmol of hCPR [23].

**Microsomal fatty acid metabolism**

1-14C-labelled AA (53 mCi/mmol), EPA (55 mCi/mmol) and LA (dodecanoic acid) (57 mCi/mmol), having radiochemical purities >99%, were purchased from Hartman Analytical. To analyse the whole range of primary and secondary metabolites produced by CYP33E2, reactions were performed in 200 μl of 100 mM potassium phosphate buffer (pH 7.2) containing 50 pmol of CYP and the respective substrates at a final concentration of 20 μM. The recombiant microsomal enzyme was pre-incubated with the substrates for 5 min at 25°C. The reactions were started with NADPH (1 mM final concentration) and terminated after 30 min of shaking at 25°C by adding 10 μl of 0.4 M citric acid. Controls included omission of NADPH from the reaction mixtures and the use of microsomes lacking CYP33E2, hCPR or both of these components.

Kinetic analysis of CYP33E2-catalysed substrate conversions was performed using EPA, AA and LA at concentrations of 5, 10, 20, 40 and 80 μM. The reactions were terminated after 5 min in order to analyse product formation in the linear range of substrate conversion and to avoid the formation of secondary metabolites. The reactions were performed in triplicate for each substrate concentration.

**Analysis of the metabolite profile**

The reaction products were extracted into ethyl acetate and analysed in a first step by RP (reversed-phase)-HPLC essentially as described previously [23–25]. Both EPA and
AA metabolites were resolved using a Nucleosil 100–5 C18 HD column (250 mm × 4 mm; Macherey-Nagel) and a linear solvent gradient of acetonitrile/water/acetate acid (50:50:0.1, by vol.) to acetonitrile/acetate acid (100:0.1, v/v) over 40 min at a flow rate of 1 ml/min [23]. LA metabolites were esterified with diazomethane and resolved with a linear gradient of acetonitrile/water/acetate acid (29.5:70:5:0.1, by vol.) to acetonitrile/water/acetate acid (59.5:40:5:0.1, by vol.) over 30 min followed by acetonitrile/acetate acid (100:0.1, v/v) at a flow rate of 1 ml/min for 15 min. [25]. Metabolites were detected and quantified using a radio flow detector. Authentic standard compounds were prepared and used as described previously [23, 24].

The regioisomeric hydroxy- and epoxy-metabolites of EPA and AA were collected from RP-HPLC and further resolved by NP (normal-phase)-HPLC according to established procedures [23]. CP (chiral-phase)-HPLC served to separate the enantiomers of 17,18-EEQ (17,18-epoxyeicosatetraenoic acid) and was carried out using a Chiralcel OB column (250 mm × 4.6 mm; Daicel) as described previously [24].

Selected metabolites with uncertain identity were analysed further by LC (liquid chromatography)–MS using the triple-quadrupole tandem mass spectrometer Agilent 6460 combined with an Agilent 1200 HPLC system. The spectrometer was equipped with a jetstream electrospray ionization source, operated in negative-ion mode. A Zorbax Eclipse Plus-C18 2.1 mm × 150 mm × 3.5 μm column was used as the stationary phase and acetonitrile/aqueous ammonium acetate solution (0.01 mol/l) as the mobile phase. Acetonitrile was increased from 5 to 95% during the first 10 min and held for a further 10 min. The injection volume was 5 μl. A single-stage MS measurement with a fragmentor voltage of 130 V was performed first to identify the molecular ion masses of the reaction products in question. Then, a product ion scan of the molecular ions was carried out at a collision energy of 10 V. The retention times and fragments in the CID (collision-induced dissociation) mass spectra were compared with known fragment patterns of this or similar compounds.

**Determination of the endogenous CYP–eicosanoid profile**

The CYP–eicosanoid profile was determined for the wild-type as well as fat-1(wa9) and fat-3(wa22) mutant strains (three independent cultures per strain). The nematodes were harvested and prepared for LC–MS/MS analysis essentially as described previously [14]. Briefly, aliquots corresponding to 30 mg of wet weight were mixed with internal standard compounds {10 ng each of 20-HETE-d6 20-[^H]hydroxyeicosatetraenoic acid), 14,15-EET-d4 (14,15-[^H]epoxyeicosatrienoic acid) and 14,15-DHET-d11 (14,15-[^H]dihydroxyeicosatrienoic acid); Cayman Chemicals] and subjected to alkaline hydrolysis followed by solid-phase extraction of the metabolites. HPLC and MS conditions as well as the multiple reaction monitoring for the analysis of the CYP–eicosanoid profile were exactly as described previously [23].

**GFP (green fluorescent protein) constructs and transgenic line**

A 1.2 kb promoter region of CYP33E2 was amplified using the Expand high-fidelity PCR system (Roche) (forward primer, 5′-GTCGAAATTTGCGGTTCTAC-3′; reverse primer, 5′-TCTGAAAGAGAAATATTTAATT-3′), cloned into Promoega’s PGEM-T vector, and re-cloned by using the SphI and XbaI/SpeI sites into the plasmid pPD97.78 (Addgene), a nematode worm expression vector containing the GFP gene. The correctness of the construct was confirmed by DNA sequencing. Young adult pha-1(e2123ts) nematodes were transfected by shooting plasmid (recombinant pPD97.78 and pha-1 wild-type allele containing pBX)–coated gold particles into the gonads using a gene gun [26]. Second generation L1-transformants expressing the wild-type pha-1 allele were selected at the restrictive temperature of 25°C. GFP expression was confirmed using a fluorescence microscope (Nikon Eclipse E200).

**Confocal microscopy**

Individually picked nematode worms were transferred on to an agarose pad (a microscope slide coated with a 200-μm-thick 3% agarose layer), immobilized by adding 50 μM tetramizole hydrochloride (Sigma–Aldrich) and finally covered with a glass coverslip. For confocal microscopy, a Bio-Rad Laboratories MRC1024 instrument with an argon–krypton laser attached to Nikon Diaphot inverted microscope was used. At least five to ten worms were analysed from four different cultivations using 10–40× magnification. Z-stacks were collected at different step sizes using the LaserSharp software. Image analysis was done using Huygens Essential software from SVI and ImageJ (NIH).

**Pharyngeal pumping rate and RNAi (RNA interference)**

On the third day of adulthood, the pharyngeal pumping rate was quantified. Nematodes of the different treatments were randomly selected (n ≥ 10) and the pumping frequency was determined three times over a 15 s timespan. For each assay, three independent experimental trials were performed. In the case of the fat-3 experiments, the bacterial food was supplemented with EPA, AA (each 1 μg/ml) or kept unchanged. The CYP33E2 RNAi by feeding experiment, as well as the attendant test of its efficiency by qRT (quantitative real-time)-PCR, following exactly the protocol described by Kulas et al. [14]. The HT115(ΔE3) feeding strain carrying the empty L4440 vector served as control. For testing the effect of CYP inhibition on the pumping rate, the nematodes were transferred to fresh plates and pre-treated for 24 h with 17-ODYA [27] or PPOH [28] (both from Cayman Chemicals via Biomol). The compounds were added directly to the bacterial food at a final concentration of 1 mM. DMSO at 0.3% served as vehicle control.

**RNA preparation**

For preparation of total RNA, 48 h nematode cultures from two 94-mm-diameter NGM agar plates were washed off with M9 buffer [18]. Nematodes were washed twice with M9 buffer and collected through gravity-mediated down-settling. Isolation of total RNA was performed with the Total RNA isolation kit (Macherey-Nagel), including removal of the genomic DNA by DNase digestion. First, the nematode pellet (approximately 100 mg of fresh weight) was resuspended in 2 vol. of the kit’s lysis buffer, supplemented with 1 vol. of glass beads (diameter 0.5 mm; Braun-Melsungen) and mechanically homogenized twice for 2 min using a SpeedMill P12 from Analytik Jena. This step was found to markedly enhance the RNA yield. All further steps were carried out following the protocol of the kit supplier exactly.

**Statistical analysis**

The pharynx activity assay datasets were analysed by one-way ANOVA to test for significant differences between treatments
followed by the Bonferroni test to identify treatments that were significantly different from the control. All statistical tests were performed using Sigma Stat 3.5 (SPSS®). The Enzyme Kinetics Module of SigmaPlot 7 (SPSS) was used for data analysis according to Michaelis–Menten kinetics.

RESULTS
Cloning and heterologous expression of CYP33E2

The CYP33E2 cDNA amplified from C. elegans N2 showed 100% identity with the corresponding mRNA (GenBank® RefSeq NM_069069) and WormBase® sequences (F42A9.5). Heterologous expression of the CYP33E2 cDNA was achieved in a baculovirus/Sf9 insect cell system. Microsomes isolated from insect cells transfected with the recombinant CYP33E2–baculovirus showed well-shaped reduced CO spectra with a Soret peak at 450 nm, indicating that the majority of the P450 enzyme expressed retained haem thiolate co-ordination in the ferrous–CO complex. Moreover, there was no evidence for any substantial amount of a P420 form, which could indicate an inactive form of the enzyme in the resting state (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350689add.htm). In contrast, control microsomes isolated after transfection with an empty baculovirus were free of any spectrally detectable CYP protein. Successful CYP33E2 expression was also revealed using SDS/PAGE by the presence of a prominent 53 kDa protein band that occurred only in the microsomal samples of cells infected with the recombinant CYP33E2–baculovirus (results not shown). Maximal expression levels of 150 nmol of CYP33E2 per litre of cell culture were obtained 48 h after infection. Co-expression of CYP33E2 and hCPR was achieved by co-infection of the Sf9 cells with the corresponding recombinant baculoviruses. The microsomes obtained from the co-infected cell cultures and used for the subsequent metabolic studies contained 0.3 nmol of spectrally active CYP33E2/mg of protein and had a CYP/hCPR ratio of approximately 5:1.

Metabolism of LA by recombinant CYP33E2

We first used LA as a test substrate to confirm the successful reconstitution of a microsomal mono-oxygenase system consisting of CYP33E2 and hCPR. As shown in Figure 1, LA was efficiently metabolized by the complete system in the presence of NADPH. No product formation occurred omitting NADPH or using microsomes lacking CYP33E2, hCPR or both of these components.

The main products of CYP33E2-catalysed LA conversion were 11- and 12-OH-LA [11- and 12-hydroxydodecanoic (‘hydroxylauric’) acid] representing nearly 90% of the total product (Figure 1A and Table 1). 11-OH- and 12-OH-LA were formed in a ratio of 5:3:1. Dodecanediacarboxylic acid, potentially co-migrating with 11-OH-LA in RP-HPLC, was not formed, as proved after converting the reaction products into the corresponding methyl esters (Figure 1B). Minor products, indicated as LX1 and LX2 in Figure 1, could not be unequivocally identified. In LC-MS, LX2 showed a molecular ion at m/z 199, suggesting that this 1–14C-labelled product originated from LA dehydrogenation.

Kinetic analysis showed that the CYP33E2/hCPR mono-oxygenase system hydroxylated LA with a V max value of 5.6 nmol/min per min and an apparent K m value of 28 μM (Table 1). The relative ratio of 11-OH-LA/12-OH-LA production was independent of the substrate concentration, and the kinetic parameters refer to the total formation of both primary products.

![Figure 1 Metabolism of LA by recombinant CYP33E2](image)

(A) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 4 nmol of LA with 50 pmol of CYP33E2 in a total volume of 0.2 ml for 30 min at 25°C in the presence of 1 mM NADPH (black line). No substrate conversion occurred in control samples, where NADPH was omitted (grey line) or where microsomes devoid of CYP33E2 or hCPR were used. The two main metabolites co-migrated with authentic 11-OH-LA (Rt = 20.4 min) and 12-OH-LA (Rt = 21.8 min). Unknown products are designated LX1 (Rt = 21.1 min) and LX2 (Rt = 40.7 min). See Table 1 for further characterization of the metabolites. (B) Representative RP-HPLC chromatograms comparing the LA-derived metabolite patterns produced by CYP33E2 (black line) and mouse Cyp4a12a (grey line). The extracted samples were treated with diazomethane to generate the corresponding methyl esters (ME) that eluted at 32.9 (11-OH-LA-ME), 33.7 (LX1-ME), 34.3 (12-OH-LA-ME), 40.9 (DODA-DME, dodecanediacarboxylic acid dimethyl ester), 42.6 (LX2-ME) and 43.9 min (LA-ME).

<table>
<thead>
<tr>
<th>Table 1 Metabolism of LA by recombinant CYP33E2</th>
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<tr>
<td>Metabolite pattern</td>
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<tr>
<td>Primary products</td>
</tr>
<tr>
<td>11-OH-LA</td>
</tr>
<tr>
<td>12-OH-LA</td>
</tr>
<tr>
<td>Other products</td>
</tr>
<tr>
<td>LX1 (unknown)</td>
</tr>
<tr>
<td>LX2 (dehydro-LA)</td>
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</table>
1-14C-labelled metabolites showed the expected molecular ions approximately 13:1 (Figure 2B). In LC-MS analysis, both co-migrated with 19-/20-HETE in RP-HPLC (Figure 2A). Further of NADPH and none of the metabolites occurred in incubations (Figure 2A). Like LA, AA was only metabolized in the presence as well as a series of secondary and unknown metabolites concentration of AA) of primary hydroxy- and epoxy-metabolites, samples, where NADPH was omitted (grey line) or microsomes devoid of CYP33E2 or hCPR by NP-HPLC. Resolution of the product peak that was eluted at 15.4 min from RP-HPLC (see 25.9 min (5,6-EET). Metabolites with uncertain or unknown identity are marked (AX1–AX5). See Table 2 for further characterization of the metabolites. (B) Resolution of 19-HETE and 20-HETE displays an informative CID spectrum with fragment compounds, the product that was eluted directly in front of 19-HETE (Rt = 15.4 min) and a series of minor reaction products were eluted between 19-/20-HETE and 14,15-EET in RP-HPLC (Figure 2A and Table 2). Typically this region contains the monohydroxy-derivatives of AA originating from CYP-catalysed subterminal and mid-chain oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations. Most of the products indeed showed corresponding oxidations.

**Table 2 Metabolism of AA by recombinant CYP33E2**

The metabolite pattern was analysed by RP-HPLC as shown in Figure 2A. 19- and 20-HETE were resolved by NP-HPLC (Figure 2B). Results are mean ± S.D. values from reactions performed with at least three independent microsomal preparations. The product distribution indicates the relative contribution of the metabolite class indicated to total product formation (bold values) and of the respective individual metabolites to a given metabolite class (non-bold values). The molecular mass was calculated from the m/z values of the molecular ions (M + 1) in LC–MS and adjusted to the products formed from unlabelled AA (−2 for the presence of 13C4 in the substrate used). The apparent K_m (46.8 ± 6.1 μM) and V_max (8.2 ± 0.4 nmol/min per nmol) values for total AA hydroxylase and epoxygenase activity of recombinant CYP33E2 were determined using short-term incubation to avoid secondary product formation as described in the Experimental section.

<table>
<thead>
<tr>
<th>Metabolite pattern</th>
<th>Product distribution (%)</th>
<th>Molecular mass (g/mol)</th>
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<tbody>
<tr>
<td>Primary products</td>
<td></td>
<td></td>
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<tr>
<td>Epoxides</td>
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<tr>
<td>14,15-EET</td>
<td>25.1 ± 3.8</td>
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</tr>
<tr>
<td>11,12-EET</td>
<td>43.3 ± 1.5</td>
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</tr>
<tr>
<td>8,9-EET</td>
<td>15.9 ± 10.1</td>
<td>320</td>
</tr>
<tr>
<td>5,6-EET</td>
<td>4.8 ± 1.1</td>
<td>320</td>
</tr>
<tr>
<td>Hydroxy products</td>
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<tr>
<td>20-HETE</td>
<td>4.7 ± 0.4</td>
<td>320</td>
</tr>
<tr>
<td>19-HETE</td>
<td>62.3 ± 3.2</td>
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<tr>
<td>18-HETE (AX2.1)</td>
<td>11.0 ± 1.6</td>
<td>320</td>
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<tr>
<td>HETE (AX2.2)</td>
<td>7.9 ± 1.3</td>
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<tr>
<td>HETE (AX3.1)</td>
<td>4.0 ± 0.5</td>
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<tr>
<td>Unknown (AX3.2)</td>
<td>1.5 ± 0.4</td>
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</tr>
<tr>
<td>HETE (AX4.1)</td>
<td>2.1 ± 0.1</td>
<td>320</td>
</tr>
<tr>
<td>Unknown (AX4.2)</td>
<td>6.5 ± 1.4</td>
<td>318</td>
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<tr>
<td>Other products</td>
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<td></td>
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<tr>
<td>AX5 (dehydro-AA)</td>
<td>100</td>
<td>302</td>
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<tr>
<td>Secondary products</td>
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<tr>
<td>HEET (AX1)</td>
<td>20.5 ± 2.0</td>
<td>336</td>
</tr>
<tr>
<td>Unidentified</td>
<td>≈13</td>
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</table>

**Figure 2 Metabolism of AA by recombinant CYP33E2**

(A) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 40 nmol of AA with 500 pmol of CYP33E2 in a total volume of 2 ml for 30 min at 25 °C in the presence of 1 mM NADPH (black line). None of the metabolites was produced in control samples, where NADPH was omitted (grey line) or microsomes devoid of CYP33E2 or hCPR were used. The main metabolites co-migrated with authentic 19/20-HETE (Rt = 15.4 min) and a set of regioisomeric monoepoxides at 23.5 (14,15-EET), 24.9 (11,12-EET), 25.3 (8,9-EET) and 25.9 min (5,6-EET). Metabolites with uncertain or unknown identity are marked (AX1–AX5). See Table 2 for further characterization of the metabolites. (B) Resolution of 19-HETE and 20-HETE by NP-HPLC. Resolution of the product peak that was eluted at 15.4 min from RP-HPLC (see A) revealed that CYP33E2 (black line) produced predominantly 19-HETE (Rt = 14.5 min), whereas 20-HETE (Rt = 20 min) was the main product in reactions samples run in parallel with mouse Cyp4a12a (grey line).

**Metabolism of AA by recombinant CYP33E2**

AA was metabolized by CYP33E2 to a complex product pattern that consisted after long-term incubation (30 min at a 20 μM concentration of AA) of primary hydroxy- and epoxy-metabolites, as well as a series of secondary and unknown metabolites (Figure 2A). Like LA, AA was only metabolized in the presence of NADPH and none of the metabolites occurred in incubations with control microsomes lacking CYP33E2.

The main primary hydroxy-metabolites produced by CYP33E2 co-migrated with 19-/20-HETE in RP-HPLC (Figure 2A). Further resolution of this product peak by NP-HPLC revealed that CYP33E2 produced 19-HETE and 20-HETE in a ratio of approximately 13:1 (Figure 2B). In LC-MS analysis, both 1-13C-labelled metabolites showed the expected molecular ions at m/z 321. Further confirming the identity of the main hydroxy-metabolite, CID yielded product ions at m/z 303 (loss of water), 275 (loss of CO2) and 231 (combined loss of CO2 and ethanol) as characteristic for 19-HETE.

The primary epoxy-metabolites produced by CYP33E2 included all four regioisomeric EETs, 14,15-EET, 11,12-EET and 8,9-EET were produced in a ratio of approximately 2.8:2:2.1. In addition, low amounts of the highly unstable 5,6-EET (approximately 5% of total epoxygenase product) were detectable (Figure 2A and Table 2). A series of minor reaction products were eluted between 19-/20-HETE and 14,15-EET in RP-HPLC (Figure 2A and Table 2). Typically this region contains the monohydroxy-derivatives of AA originating from CYP-catalysed subterminal and mid-chain oxidations. Most of the products indeed showed corresponding molecular ions at m/z 321 in LC–MS (Table 2). Among these compounds, the product that was eluted directly in front of 19-/20-HETE displayed an informative CID spectrum with fragment ions at m/z 303 (loss of water) and 263 (loss of propan-1-ol) indicating the presence of 18-HETE. In addition, there were two minor products showing molecular ions at m/z 319, suggesting further oxidation of hydroxy- to oxo-derivatives (Table 2). The uncertain metabolite ‘AX5’ that was eluted 3.5 min before AA (compare Figure 2A) showed a molecular ion at m/z 303 and thus most probably represented a dehydrogenation product of AA.

Secondary products, whose concentrations strongly increased upon prolonged reaction times, were eluted between 8.6 and 10 min in RP-HPLC (Figure 2A). LC–MS revealed the presence of multiple compounds with a common molecular ion at m/z 337, indicating that these metabolites comprised dioxygenated products. The most likely candidates are regioisomeric HEETs (hydroxyepoxyeicosatrienoic acids) formed by secondary epoxidation of 19- and 20-HETE or by secondary hydroxylation.

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of EETs. Confirming this assumption, 19-/20-HETE was converted by CYP33E2 into products with retention times identical with those of the secondary AA metabolites (results not shown). DHETs, the hydrolysis products of EETs, were eluted between 12.5 and 14.5 min from RP-HPLC and were not formed under the conditions used.

During short-term incubations (5 min), the secondary products described above were not formed and the relative ratio of primary hydroxy- and epoxy-metabolites was 1.4:1, independent of the substrate concentration. Formation of total primary hydroxy- and epoxy-metabolites from AA followed Michaelis–Menten kinetics with an apparent $K_m$ value of approximately 47 $\mu$M and a $V_{\text{max}}$ value of 6 nmol/min per nmol (Table 2).

Metabolism of EPA by recombinant CYP33E2
At a substrate concentration of 20 $\mu$M, CYP33E2 metabolized EPA at a rate almost 2-fold higher than that of AA. The complex product pattern obtained after long-term incubation (30 min) consisted predominantly of monohydroxy- and monooxyo-derivatives (Figure 3A and Table 3). On the basis of co-migration with authentic standard compounds, the primary hydroxy-metabolites detectable in RP-HPLC at $R_t$ (retention time) 14.5 min were 19- and/or 20-HEPE (19- and/or 20-hydroxyeicosapentaenoic acid) (Figure 3A). Further resolution by NP-HPLC demonstrated that this fraction consisted almost exclusively of 19-HEPE, whereas 20-HEPE was not detectable at all (Figure 3B). In LC–MS, the major metabolite isolated from NP-HPLC showed a molecular ion at $m/z$ 319 and CID-induced fragment ions at $m/z$ 301 (loss of H$_2$O), 273 (loss of $^{14}$CO$_2$), and 227 (combined loss of $^{14}$CO$_2$ and ethanol) as characteristic for 19-HEPE.

17,18-EEQ represented almost 70% of the total epoxy-metabolites produced by CYP33E2 from EPA (Figure 3A and Table 3). CP-HPLC revealed a moderate stereoselectivity of CYP33E2 in favour of producing the $R,S$-enantiomer of 17,18-EEQ (Figure 3C). Other regiosomeric EEQs migrating largely
The endogenous CYP–eicosanoid profile of *C. elegans*

To prove the *in vivo* formation of CYP-dependent eicosanoids, we analysed *C. elegans* cultures by LC–MS/MS for the presence of EPA- and AA-derived monohydroxy- and monoepoxy-metabolites. As summarized in Figure 4, wild-type nematode worms contained a set of well-detectable metabolites that was clearly dominated by the epoxygenase and hydroxylase products of EPA. The total EPA/AA metabolite ratio was approximately 95.5 (for the complete dataset, see Supplementary Table S1 at http://www.BiochemJ.org/bj/435/bj4350689add.htm). Cultures of wild-type nematodes as well as of fat-1(wa9) and fat-3(wa22) mutant strains were harvested and the nematodes were analysed by LC–MS/MS for the presence of AA- and EPA-derived metabolites. The bars showing the total amounts of hydroxy-metabolites derived from AA (HETE) or EPA (HEPE) represent the sum of the corresponding n- and (n−1)-hydroxy-products. The bars designated EET and EEO show the total amounts as well as the regioisomeric composition of the epoxygenase products. Since the nematodes contained not only a set of regioisomeric epoxides, but also a set of the corresponding diols, the total epoxygenase products are given as the sum of the respective primary and secondary metabolites. Results are means+s.D. from three independent cultures performed for each strain. For the complete dataset, see Supplementary Table S1 (at http://www.BiochemJ.org/bj/435/bj4350689add.htm).

The endogenous CYP–eicosanoid profile of *C. elegans*
The pharynx of *C. elegans* pumps continuously and we measured an average frequency of 256 ± 10 contractions/min in wild-type nematodes at the third day of adulthood (Figure 7A). *fat-3*(wa22) mutant nematodes cultured in parallel showed a significantly reduced pumping rate of 199 ± 13 contractions/min (mean ± S.E.M. from 30 individual nematodes per group; *P* < 0.001 compared with wild-type control) that was restored almost to the wild-type level by AA/EPA supplementation.

Figure 5  **CYP33E2 promoter-driven GFP expression in *C. elegans***

Transgenic nematodes carrying the pCYP33E2::GFP construct were transferred on to an agarose-pad-mounted microscope slide and covered by a glass cover slip. (A) Gravid hermaphrodite and corresponding GFP fluorescence. (B) Anatomy of the pharynx, schematic of a lateral view, anterior to the left. The Figure is based on reconstructions made by different authors [30–32], which were originally derived from electron micrographs presented by Albertson and Thomson [29]. (C) Four representative confocal images representing the 13th, 21st, 30th and 39th horizontal section plane of total 81 images from a nematode pharynx (left), the corresponding GFP fluorescence (centre) and a merge of the two images (right). The monitoring level runs top down as indicated. Expression was highest in the pro- and meta-corpus and extended to the isthmus and terminal bulb region of the pharynx. The white arrows mark fluorescent cell structures which surround the buccal cavity and probably do correspond to epithelial cells.

### In vivo localization of CYP33E2

To identify the expression sites of CYP33E2 in *C. elegans*, we used nematode worms stably transfected with a pCYP33E2::GFP reporter construct. As shown in Figure 5(A), CYP33E2 promoter-driven expression of the GFP occurred exclusively in the pharynx and, not visible in each individual, in the pharyngeal-intestinal valve of the nematodes. This type of strong pharynx-restricted expression was observed throughout larval development and in adult nematodes. Expression was most prominent in the pharyngeal pro- and meta-corpus (Figure 5B). Confocal imaging suggested marginal, muscle, and/or epithelial cells as the major expression sites of the pCYP33E2::GFP construct within the pharynx (Figure 5C). Radially, only marginal cell types are continuously organized with three-fold symmetry around the pharyngeal lumen. Imaginary cross sections derived from confocal imaging series of the pro- and meta-corpus (Figure 6B) indicated that the GFP reporter was expressed in the three marginal mc1 cells, but not in the pm3 and pm4 muscle cells, which should be visible in a cross-section rather as a six-fold-divided structure (Figure 6C). A further labelling of the mc2 and mc3 marginal cells in the isthmus and terminal bulb becomes visible in Figure 5(C) (see the two lower fluorescence images). Marginal cells have characteristics of epithelia and are coupled to pharyngeal muscles via gap junctions and large interlocking extensions [29,30]. The finger-like fluorescent structures shown in Figure 6(A) obviously represent these interlocking extensions that hold marginal cells to muscles. Furthermore, an expression of pCYP33E2::GFP also in the epithelial e1, e2, and e3 cells seems most likely, as shown both by fluorescent cell structures around the buccal cavity (Figure 5C, see the arrows in the two upper fluorescence images, similar structures are also visible in Figure 6A) and by further fluorescence additionally to the three-fold symmetry of marginal cells appearing in the second cross-section of Figure 6(B). The latter might also correspond to pm2 muscle cells (Figure 6C).

### Lack of LC-PUFAs, CYP33E2 gene silencing and CYP inhibitors decrease the pharyngeal pumping frequency

The pharynx of *C. elegans* pumps continuously and we measured an average frequency of 256 ± 10 contractions/min in wild-type nematodes at the third day of adulthood (Figure 7A). *fat-3*(wa22) mutant nematodes cultured in parallel showed a significantly reduced pumping rate of 199 ± 13 contractions/min (mean ± S.E.M. from 30 individual nematodes per group; *P* < 0.001 compared with wild-type control) that was restored almost to the wild-type level by AA/EPA supplementation.

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**Figure 6 Analysis of CYP33E2 promoter-driven GFP expression in the pharyngeal pro- and meta-corpus of *C. elegans***

(A) A pair of confocal images representing the 34th horizontal section plane of total 51 images and the merge of both images. Note the finger-like extensions which are suggested to hold the marginal cells to muscles as clearly visible in the central fluorescence image. (B) Three derived vertical sections based on the 51 horizontal fluorescent images, the selected positions of imaginary sectioning are highlighted by white arrows in (A). (C) The corresponding schemata of pharyngeal cross sections at the same position: white, pharyngeal lumen; blue, muscle cells (pm3, pm4); green, marginal cells (mc1); lilac, epithelial cells (e1–e3); yellow, muscle cells (pm2); grey, motor neurons; red, interneurons. These simplified schemata were derived from sections of the ‘Slidable Worm’ as presented by WormAtlas (http://www.wormatlas.org/SW/SW.php, slices 19, 34 and 50). Note that the three GFP-expressing cells visible in (B) reflect the three-fold symmetry of the marginal mc1 cells.
CYP-dependent EPA metabolism in Caenorhabditis elegans

Figure 7  Pharyngeal pumping activities of *C. elegans* N2 as modulated by fat-3 mutation, CYP33E2 gene silencing and pharmacological inhibition of CYP activity

(A) The nematodes were monitored on the third day of adulthood. Shown are the contractions/min (*n* = 30–60 per group); for each individual animal, the pumping frequencies were counted in triplicate. Results are presented as box plots showing the 5th/95th percentile; the continuous line corresponds to the median; the broken line shows the mean. *P* < 0.05, **P** < 0.001 (one-way ANOVA). (B) The CYP33E2 mRNA level decreased in response to RNAi feeding. Total RNA was isolated from *C. elegans* cultures fed on bacteria either containing the CYP33E2 RNAi vector or the empty control vector. qRT-PCR was performed using primer pairs specific for the amplification of CYP33E2 and act-1 (actin-1 gene, as housekeeper reference). Shown are the threshold cycles in dependency of the starting concentrations varied by a four-step 1:5 dilution series. Empty squares show the control; filled circles correspond to the CYP33E2 RNAi sample. E, estimated PCR efficiency; int, intercept.

Figure 7 (A). RNAi feeding efficiently reduced the CYP33E2 mRNA level. Approximately 30% of the CYP33E2 mRNA remained compared with the empty vector feeding control, as indicated by a mean delay of 1.72 cycles in CYP33E2-specific qRT-PCR and unchanged amplification of the reference act-1 mRNA (Figure 7B). Silencing of CYP33E2 expression resulted in a decrease in the pumping rate to 226 ± 10 (*P* < 0.01 compared with empty vector feeding control) (Figure 7A). Similarly, *C. elegans* cultures pre-treated with 17-ODYA or PPOH for 24 h displayed significantly reduced pumping rates of 225 ± 14 and 226 ± 13 (each *P* < 0.05 compared with vehicle control) (Figure 7A).

DISCUSSION

The present study revealed that CYP33E2 is specifically expressed in the pharynx of *C. elegans* and demonstrates that this CYP isoform functions as an epoxygenase and *n* − 1 hydroxylase of EPA and AA. Moreover, our results suggest a role for CYP33E2 in the regulation of pharyngeal activity.

CYP33E2 shares with other microsomal CYP isoforms a series of characteristic sequence elements including the N-terminal signal anchor-, central helix I- and C-terminal haem-binding regions. In particular, CYP33E2 shows pronounced amino acid sequence homology with mammalian AA epoxygenases belonging to the CYP2C and CYP2J subfamilies [14]; for an alignment with human CYP2J2, see Supplementary Figure S2 (http://www.BiochemJ.org/bj/435/bj4350689add.htm). These structural similarities are now complemented by our functional studies showing that CYP33E2 can be reconstituted with the human NADPH-CYP reductase to an active microsomal mono-oxygenase system that metabolizes EPA and AA. The metabolite pattern produced by CYP33E2 closely resembles that of CYP2J2, an epoxygenase highly expressed in the human heart [26,34,35].
Like CYP2J2, CYP33E2 attacks all four double bonds of AA to produce regioisomeric EETs and predominantly epoxidizes the n−3 double bond to produce 17,18-EEQ as the main metabolite from EPA. Moreover, both CYP2J2 and CYP33E2 hydroxylate AA and EPA with a high regioselectivity in favour of producing 19-HETE and 19-HEPE respectively. Furthermore, LA was mainly hydroxylated at the n−1 position. These enzymatic features largely exclude a role for CYP33E2 in fatty acid degradation and rather point to a role for this enzyme in the production of lipid mediators, similar to that increasingly recognized for its mammalian counterparts [8,9,36,37].

EPA- and AA-derived epoxy- and hydroxy-metabolites were clearly detectable as endogenous constituents in cultured wild-type nematode worms, indicating that these metabolites are also formed under in vivo conditions. The endogenous CYP-eicosanoid profile showed a remarkable similarity to the metabolite pattern produced by recombinant CYP33E2. Both in vivo and in vitro, 17,18-EEQ and 19-HEPE were the main metabolites derived from EPA. Moreover, the similarity extends to the pattern of regioisomeric EETs produced from AA. However, we also observed some major differences. CYP33E2 produced 19- and 20-HETE at a ratio of 13:1, whereas the cultured nematodes contained these metabolites in almost equal amounts. Furthermore, 20-HEPE, a metabolite not produced by CYP33E2, was clearly present as an endogenous constituent. This comparison suggests a major role for CYP33E2 in the formation of EPA and AA metabolites in C. elegans, but also indicates that further CYP isoforms contribute to the endogenous metabolite pattern. In particular, the expression of at least one further CYP isoform that, unlike CYP33E2, predominantly functions as ω-hydroxylase has to be assumed. We observed huge differences comparing the endogenous CYP-eicosanoid profile of the wild-type strain with that of the fat-1 and fat-3 mutant strains. The wild-type strain contained predominantly EPA-derived metabolites, the fat-1 strain expressed almost exclusively AA-derived metabolites, and the fat-3 strain was essentially devoid of any EPA- and AA-derived metabolites. These differences in endogenous metabolite formation fitted perfectly to the relative bioavailability of the precursor fatty acids: EPA is the predominant LC-PUFA in the wild-type strain [14], the fat-1 strain is unable to produce EPA, and the fat-3 strain is deficient in the biosynthesis of both EPA and AA. Considering the substrate specificity of CYP33E2, this enzyme presumably contributed not only to the formation of EPA metabolites in the wild-type strain, but also of the AA metabolites in the fat-1 strain. By analogy to these findings, the endogenous CYP-eicosanoid profile of mammalian tissues also largely depends on the relative bioavailability of the various LC-PUFAs that may serve as alternative CYP substrates [23,38].

The pharynx of C. elegans is a rhythmically active muscular pump that sucks nutrients (bacteria) and passes them to the intestine of the animal [39,40]; pharyngeal pumping is mainly excited by the motor neurons MC and M3 [29]. Our results indicate that CYP33E2 is predominantly expressed within the three marginal mc1 cells that extend from the most anterior muscle cell in the procorpus down to the nerve ring in the metacorpus of the pharynx. Weak, but significant, expression was also found in the subsequent isthmus and terminal bulb region, where CYP33E2 is mainly localized in the marginal mc2 and mc3 cells. In each of these three segments, the marginal cells are coupled to pharyngeal muscle cells via gap junctions. Marginal cells are hypothesized to function as relay stations to synchronously transmit signals from motor neurons to the muscle cells and thus to support co-ordinated contraction of the pharynx [39]. Marginal cells might be also involved in generating the spontaneous rhythmic activity of the pharynx that remains even after complete ablation of the pharyngeal nervous system [31,40].

The present study shows that RNAi-mediated silencing of CYP33E2 expression as well as pharmacological inhibition of CYP activity causes a moderate, but significant, decrease in the pharyngeal pumping rate. Reduced pharyngeal activity may result in caloric restriction which in turn can be associated with increased lifespan demonstrated by a series of the so-called ‘eat-mutants’ of C. elegans [41–43]. Indeed, there is one report showing that CYP33E2 belongs to a list of genes whose silencing results in a longevity phenotype. Specifically, RNAi-mediated gene silencing of CYP33E2 increased the mean lifespan of C. elegans by 18% [44]. Thus CYP33E2 might play an important role in the regulation of pharyngeal activity throughout the whole lifetime of the animal.

The extent of pharyngeal activity inhibition by CYP33E2 gene silencing or pharmacological CYP inhibition was similar to that observed with the fat-3(wa22) mutant. Since fat-3 mutants are unable to produce EPA and AA and CYP33E2 metabolizes these LC-PUFAs, it is tempting to speculate that the epoxy- and hydroxy-derivatives of EPA and AA may serve as mediators in the regulation of pharyngeal activity. All of our results are from nematode worms that had full access to bacterial food and were thus pumping with almost maximal and constant rates. This may explain the rather moderate effects of LC-PUFAs on CYP33E2 deficiency and it remains to future studies to define the physiological context and significance of CYP-eicosanoid formation and action for the regulation of pharyngeal activity. By analogy to the formation of CYP-eicosanoids in mammalian systems, it can be expected that CYP33E2 becomes active in response to extracellular signals that trigger the activation of phospholipases A2 and thus make free AA and/or EPA accessible to the enzyme. Potential candidates may be among the factors that regulate the feeding behaviour of C. elegans such as serotonin, acetylcholine or glutamate [40]. Another important open question concerns the identity of the final effectors of CYP-eicosanoid action. In mammals, EETs and EEQs produced in endothelial cells regulate vascular contractility by modulating the activity of large conductance- and ATP-sensitive K+ channels in the underlying vascular smooth muscle cells [39,45]. In cardiomyocytes, L-type Ca2+ channels and ATP-sensitive K+ channels are the main effectors of EET and EEQ action [46,47]. In C. elegans, leading candidates may be ion channels that shape or modulate the action potential of the pharyngeal muscle cells such as the L-type Ca2+ channel EGL-19 and the K+ channel EXP-2 [48,49]. A precedent to think about alternative mechanisms is provided by some vascular beds, where EETs activate endothelial transient receptor potential potential channels causing membrane hyperpolarization that is transferred to the smooth muscle via gap junctions [50].

Numerous studies revealed functional similarities between the C. elegans pharynx and the vertebrate heart that are considered to reflect either true orthology or convergent evolution [39,48]. The present study indicates that the apparent homologies may extend to the role for CYP-eicosanoids in the regulation of myogenic activity.

AUTHOR CONTRIBUTION

Mandy Kosel did the heterologous co-expression and conducted the first part of the HPLC analyses. Waltraud Wild conducted the second part and performed the pumping assay, and both wrote parts of the paper. Alexandra Bell generated the GFP transgenic nematode strain. Michael Rothe conducted all LC–MS/MS analyses. Carsten Lindschau performed the localization experiments using confocal fluorescence microscopy and generated the cross-sections. Christian Steinberg partially supervised Waltraud Wild and Alexandra Bell.
and carried out a final revision of the paper. Wolf-Hagen Schunck and Ralph Menzel developed the ideas of this work, supervised the practical work of Mandy Kosel, Waltraud Wild and Alexandra Bell and wrote the final version of the paper.

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SUPPLEMENTARY ONLINE DATA

Eicosanoid formation by a cytochrome P450 isoform expressed in the pharynx of Caenorhabditis elegans

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Figure S1 Spectral properties of CYP33E2 heterologously expressed in Sf9 insect cells

Typical reduced CO difference spectra of microsomal samples (1.75 mg of protein/ml) isolated 48 h after infection of the cell cultures with either empty control (grey line) or recombinant CYP33E2 baculovirus (black line).

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email ralph.menzel@biologie.hu-berlin.de).
The putative membrane anchor, the PPGP motif at its end, Helix I and the haem-binding (HR2) region are highlighted.

**Table S1** Endogenous metabolites of AA and EPA in *C. elegans*

Results are means ± S.D. (*n = 3*), expressed in ng/g.

(a) EPA-derived metabolites

<table>
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<tr>
<th>Strain</th>
<th>20-HEPE</th>
<th>19-HEPE</th>
<th>17,18-EEQ</th>
<th>17,18-DIHEQ</th>
<th>14,15-EEQ</th>
<th>14,15-DIHEQ</th>
<th>11,12-EEQ</th>
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<th>8,9-DIHEQ</th>
<th>5,6-EEQ</th>
<th>5,6-DIHEQ</th>
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<tr>
<td>N2</td>
<td>42.2 ± 4.4</td>
<td>256.7 ± 112.9</td>
<td>269.4 ± 102.6</td>
<td>521.4 ± 126.5</td>
<td>130.5 ± 8.9</td>
<td>65.7 ± 16.4</td>
<td>76.2 ± 13.0</td>
<td>24.2 ± 6.9</td>
<td>28.3 ± 5.6</td>
<td>17.1 ± 4.8</td>
<td>18.6 ± 5.8</td>
<td>10.5 ± 3.7</td>
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<tr>
<td>fat-1(wa9)</td>
<td>–</td>
<td>–</td>
<td>6.2 ± 4.7</td>
<td>16.5 ± 5.0</td>
<td>2.9 ± 2.4</td>
<td>1.0 ± 0.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>fat-3(wa22)</td>
<td>–</td>
<td>–</td>
<td>1.9 ± 0.5</td>
<td>6.9 ± 3.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.9</td>
<td>–</td>
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(b) AA-derived metabolites

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<tr>
<td>N2</td>
<td>27.6 ± 15.0</td>
<td>185.9 ± 9.9</td>
<td>3.3 ± 2.1</td>
<td>1.0 ± 0.3</td>
<td>1.8 ± 0.8</td>
<td>1.0 ± 0.5</td>
<td>2.4 ± 1.3</td>
<td>1.1 ± 0.3</td>
<td>1.8 ± 2.0</td>
<td>0.9 ± 0.4</td>
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<tr>
<td>fat-1(wa9)</td>
<td>21.4 ± 33.2</td>
<td>303.4 ± 72.7</td>
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<td>20.7 ± 6.4</td>
<td>49.8 ± 5.6</td>
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<td>16.9 ± 5.1</td>
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<td>fat-3(wa22)</td>
<td>1.7 ± 0.7</td>
<td>5.8 ± 13.6</td>
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</tr>
</tbody>
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**Figure S2** Amino acid sequence alignment of human CYP2J2 (NP_000766.2) and CYP33E2 from *C. elegans* (NP_501470)

The putative membrane anchor, the PPGP motif at its end, Helix I and the haem-binding (HR2) region are highlighted.