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Evolutionary analysis of the carnitine- and choline acyltransferases suggests distinct evolution of CPT2 versus CPT1 and related variants

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ABSTRACT

Carnitine/choline acyltransferases play diverse roles in energy metabolism and neuronal signalling. Our knowledge of their evolutionary relationships, important for functional understanding, is incomplete. Therefore, we aimed to determine the evolutionary relationships of these eukaryotic transferases. We performed extensive phylogenetic and intron position analyses. We found that mammalian intramitochondrial CPT2 is most closely related to cytosolic yeast carnitine transferases (Sc-YAT1 and 2), whereas the other members of the family are related to intraorganellar yeast Sc-CAT2. Therefore, the cytosolically active CPT1 more closely resembles intramitochondrial ancestors than CPT2. The choline acetyltransferase is closely related to carnitine acetyltransferase and shows lower evolutionary rates than long chain acyltransferases. In the CPT1 family several duplications occurred during animal radiation, leading to the isoforms CPT1A, CPT1B and CPT1C. In addition, we found five CPT1-like genes in *Caenorhabditis elegans* that strongly group to the CPT1 family. The long branch leading to mammalian brain isoform CPT1C suggests that either strong positive or relaxed evolution has taken place on this node. The presented evolutionary delineation of carnitine/choline acyltransferases adds to current knowledge on their functions and provides tangible leads for further experimental research.

1. Introduction

The carnitine/choline acyltransferase family is a family of enzymes that play diverse roles, ranging from (the regulation of) energy and fuel metabolism in mitochondria and peroxisomes to the generation of the neurotransmitter acetylcholine. In mammals, this family consists of seven transferases that catalyse the reversible transfer of acyl moieties from acyl-CoA (short-chain acyl-CoAs like acetyl-CoA; medium-chain acyl-CoAs like octanoyl-CoA, and long-chain acyl-CoAs like palmitoyl-CoA) to carnitine or choline [1–5]. A common effect of this catalytic capacity is the buffering of cellular levels of free coenzyme A (HSCoA). The transferases are different from each other regarding their physiological function, location, kinetics and substrate specificity, but have a presumed common ancestral gene from which all genes encoding these proteins have evolved [3,6] (for the carnitine transferases this has been reviewed, choline-carnitine transferase relations were also known by then [3]). These genes are only present in eukaryotes and have no prokaryotic equivalent with carnitine- or choline transferase activity, however, distant relations are known to exist between this eukaryotic family and bacterial enzymes like chloramphenicol acetyltransferase [7].

The carnitine palmitoyltransferases (CPT) 1 and 2 are traditionally known to be located at the outer and inner mitochondrial membrane, respectively, where they transesterify medium- and long chain acyl-CoAs (Fig. 1). Together with a carnitine/acylcarnitine transporter, CPT1 and 2 facilitate the net transport of fatty acyl-CoA across the mitochondrial inner membrane [2]. This carnitine shuttle enables the oxidation of fatty acids within the mitochondrial matrix. The mammalian expression of CPT2 is ubiquitous [2] and tissue specificity of the carnitine shuttle is hence provided by tissue specific expression of CPT1. Although CPT1 and CPT2 must have co-evolved to establish carnitine shuttling, the genes encoding these enzymes are the most distantly related of the carnitine and choline acyl transferases [6]. In fact, a number of databases, including Ensembl, consider CPT2 and its orthologues as a separate gene set compared to the rest of the carnitine and choline acyltransferases.

In mammals, three different CPT1 protein isoforms exist, namely CPT1A (liver-type), CPT1B (muscle-type) and CPT1C (brain-type) [8].

Abbreviations: AMPK, AMP-activated protein kinase; cChAT, common form of choline acetyltransferase; ChAT, choline acetyltransferase; CPT, carnitine palmitoyltransferase; CrAT, carnitine acetyltransferase; CrOT, carnitine octanoyltransferase; HSCoA, free coenzyme A; pChAT, peripheral form of choline acetyltransferase; TM, transmembrane

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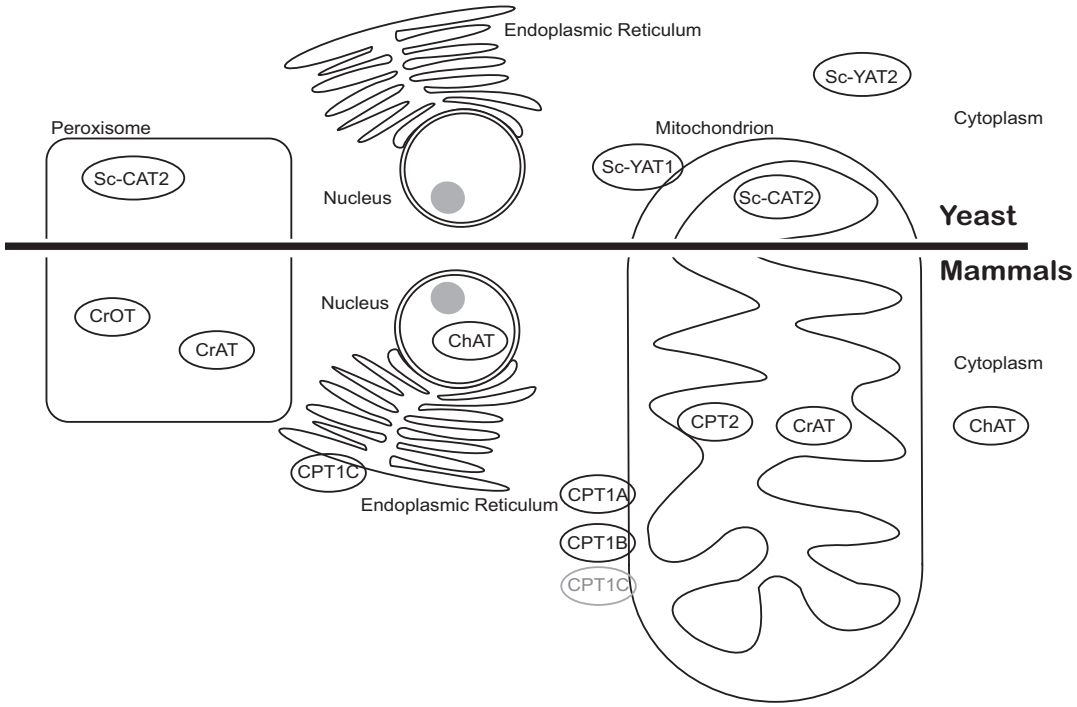

Fig. 1. Location of the carnitine and choline acyltransferases in yeast (denoted with Sc-) and mammals. In yeast (top), the three carnitine/choline acyltransferases are Sc-YAT1, Sc-YAT2 and Sc-CAT2, of which Sc-YAT1 and Sc-YAT2 are located in the cytosol, and Sc-CAT2 in peroxisomes and mitochondria. During evolution, the carnitine/choline acyltransferases have evolved from 2 enzymes in yeast to 7 enzymes in mammals (bottom). The established mitochondrial location of CPT1C is in light grey since, no mitochondrial activity of the enzyme has been established.

The CPT1 isoforms are encoded by three different genes on different chromosomal locations. Based on compiled physical [7], biochemical [9] and cytological [10] evidence, it is thought that CPT1 has a N-terminal domain consisting of two transmembrane regions and a short connecting loop, and a large catalytic C-terminal domain containing both the catalytic site and the malonyl-CoA binding site, as malonyl-CoA inhibits the CPT1 enzymes [11]. Both the N-terminus and the C-terminal domain of CPT1 enzymes are projected into the cytosol [12,13]. Because of the homology, it is thought that the proposed tertiary structure applies to all three isoforms of CPT1. However, unlike CPT1A and CPT1B, which are localized in mitochondria, CPT1C is mainly localized in the ER [8,14]. Whereas CPT1A and CPT1B control the rate of mitochondrial fatty acid oxidation, it is likely that CPT1C has another physiological function. It is hypothesized that ghrelin induces CPT1C to synthesize hypothalamic ceramides, enhancing the expression of orexigenic neuropeptides leading to food intake [15]. In addition, CPT1C is involved in spatial learning and motor function, and the regulation of the peripheral metabolism (reviewed in Casals et al. [16]). Recently, Lopes-Marques et al. [17] reassessed the evolutionary relationships of the CPT1 genes and found that CPT1C is not only present in mammals, but also in other phyla like teleosts, amphibians and coelacanth.

Not only CPT1 and CPT2, but also carnitine octanoyltransferase (CrOT) transesterifies medium- and long-chain acyl-CoAs, although this enzyme is located in peroxisomes rather than mitochondria. Its function is to enable the transport of octanoyl-CoA derived from the ω-oxidation of very-long-chain and branched-chain fatty acids [1,18,19]. Octanoylcarnitine is able to be shuttled into mitochondria for further oxidation.

Carnitine acetyltransferase (CrAT) transesterifies short-chain acyl-CoAs, and is located both in mitochondria and peroxisomes. In yeasts and mammals this protein is the product of a single gene [20,21] and the sorting to different organelles is evolutionary conserved, although the mechanism of differential sorting is quite different [21–23] (reviewed by van der Leij et al. [6]). The yeast Sc-CrAT is expressed by Sc-CAT2 (Fig. 1) [24]. However, two more genes responsible for acetyltransferase activity have been described [25,26]. These transferases, Sc-YAT1 and Sc-YAT2, are located in the cytosol (Fig. 1). Apart from these three acetyltransferases, of which orthologues exist in other fungi, no other fungal carnitine or choline acyltransferases have been described.

Finally, choline acetyltransferase (ChAT) is located in the nucleus and cytoplasm of cholinergic neurons of the central and peripheral nervous systems [27,28]. This is the only transferase within this family that transfers acetyl-CoAs to choline instead of carnitine, which results in the formation of the neurotransmitter acetylcholine (the function of acetylcholine in the central nervous system is reviewed by Oda et al. [29]). Cholinergic neurons are involved in the regulation of memory, learning, motor function and in the control of several visceral functions [30–32]. Low ChAT activity has been associated with a number of neurodegenerative diseases [29]. Two isoforms of ChAT exist. Next to the common form of ChAT (cChAT), a second isoform has been discovered (pChAT), which is mainly expressed in the peripheral nerve tissue [33]. In the cell, cChAT is mainly present in the cytosol, but can also bind to the plasma membrane and localize to the nucleus, whereas pChAT is only present in the cytosol [34]. Both isoforms are encoded by the same sequence, but the isoform cChAT (640 amino acids) results from conventional splicing, whereas pChAT (430 amino acids) results from alternative splicing by exon skipping (exon 6–9). In mammals, the nine amino acid residues necessary for binding choline and acetyl-CoA are present in both isoforms, but in pChAT the amino acid residue histidine involved in the catalytic centre is lost during exon skipping [33,34]. However, both isoforms have ChAT activity, that of pChAT being lower than cChAT [35]. Since acetylcholine is also involved in non-neuronal functions [34], it needs to be clarified whether and to what extend pChAT is involved in cholinergic neurotransmission. The expression of pChAT is conserved during evolution, since it is widely spread among invertebrate and vertebrate phyla [33,34,36]. This stresses the importance of the cholinergic system in peripheral tissues.

The substrates carnitine (L-3-hydroxy-4-*N,N,N*-trimethylaminobutyrate, C7H15NO3) and choline (2-hydroxy-*N,N,N*-

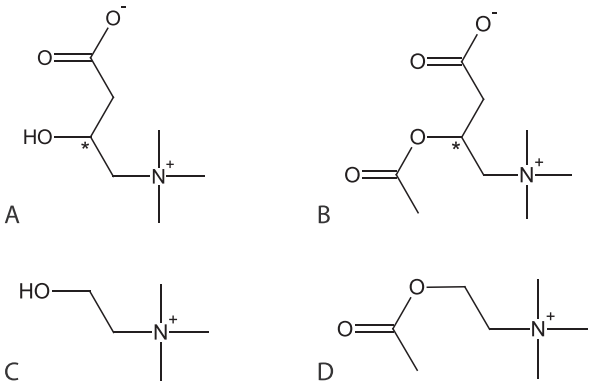

Fig. 2. Structural formulas of (A) L-carnitine, (B) acetyl-L-carnitine, (C) choline and (D) acetylcholine. The asterisk indicates the asymmetric carbon atom. Structural formulas were drawn using ChemSketch (www.actlabs.com).

trimethylethaneaminium, C5H14NO) are quite similar (Fig. 2). The major difference being the presence of a carboxyl unit in carnitine, and hence a quaternary carbon in position 3. Of the stereoisomers that are possible, the L-enantiomer of carnitine is the biologically active one. Although carnitine- and choline acyltransferases are not known for prokaryotes, both the substrates carnitine and choline are present in bacteria where they serve as osmo-, thermo- and cryoprotectants, and also can be metabolized as nutrient sources (reviewed by Meadows and Wargo [37]). Choline can be synthesized by bacteria, but this has never been demonstrated for carnitine [37,38].

The aim of this paper is to get a deeper insight in the origin and evolution of the carnitine/choline acyltransferase family in order to better understand form and function of these physiologically important enzymes. The focus will be on the analysis of (a) the Sc-YAT1-related origin and evolution of CPT2 compared to the other members of the family, which are Sc-CAT2-related; (b) the phylogenetic position of ChAT within this family; (c) the finding of several CPT1-like genes in *Caenorhabditis elegans*; and (d) the peculiar evolution of mammalian CPT1C. Evidence will be provided that (a) during evolution the animal proteins CPT2 and CPT1 switched their location from the mitochondrial matrix to the mitochondrial outer membrane and vice versa; (b) ChAT is most closely related to CrAT and shows higher protein conservation throughout evolution than the long chain acyltransferases; (c) five extra genes in *C. elegans* exist with characteristic features resembling CPT1; and (d) a particular long branch leading to mammalian CPT1C suggests either strong positive or relaxed evolution on this node and indicates elevated evolution of CPT1C.

2. Material and methods

2.1. Collection of genes

The amino acid and nucleotide sequences of the carnitine/choline acyltransferase family were collected from the Ensembl (release 85–90) and NCBI (release 215–220) databases. Sequences were retrieved from the taxa mammals, reptiles, birds, amphibians, lobe finned fishes, fishes, insects, nematodes and yeast (outgroup taxon), representing a balanced phylogenetic representation of the different classes of animals (Supplemental Tables S1 and S2). It was aimed to have full-length and error free sequences. For each protein sub-family initially amino acid alignments were made with MAFFT (v7.271) [39] using the parameters: -maxiterate 1000 and –localpair followed by visual inspection to detect any peculiar deviation in the sequences. Sequences that displayed clear deviation from the consensus were checked manually in the Ensembl/NCBI genome browsers for signs of incomplete annotation (e.g. missing start codon, missing exons, partially missing genome sequences). In case of incomplete annotation, an alternative sequence from a closely related phylogenetic taxon was searched with Blast [40] against the NCBI non-redundant database. This resulted in a list of 91 full-length error-free protein sequences. The Coelacanth CPT1C (accession XP_008111818) which shows a putative incorrect amino acid sequence annotation of position 93 to 135 and Coelacanth CPT1B (accession ENSLACP00000008053) which misses the first part of the protein were also included as Coelacanth represent a unique branch in the vertebrate tree. Thus in total 93 protein sequences were used for the phylogenetic analysis. However, for the detailed molecular phylogenetic analysis of CPT1, only the full-length and error free protein sequences were used (32 sequences). For clarity, all yeast (outgroup/crown group) annotations were preceded with Sc-.

2.2. Phylogenetic analysis

Protein and DNA alignments were made with MAFFT (v7.271) [39] using the parameters: -maxiterate 1000 and -localpair. All other options were set to default. The nucleotide alignment was adjusted manually according to the protein alignment. Alignment positions with indels in more than 3 sequences were excluded from phylogenetic analysis.

Phylogenetic maximum likelihood analyses were performed with IQ-TREE [41] (version 1.5.5 build for Linux) and MrBayes [42] (version 3.2.7-svn (r1079) ×64 build for Linux). ModelFinder [43] in IQ-TREE was used to find the optimal model of sequence evolution for each dataset (Supplemental Table S3) and maximum likelihood phylogenetic trees were constructed using default settings of IQ-TREE with 100 non-parametric bootstrapping to assess node confidences. MrBayes analysis included 2 million generations, sampling frequency every 500 generations, 2 times 4 chains and a random starting tree. For the consensus tree the first 1000 samples (25%) were discarded ("burn in" procedure) and it was manually checked if stationarity had been reached with a split frequency lower than 0.01 between the two runs. The algorithms bp-RELL [44], p-KH [45], p-SH [46], c-ELW [47] and p-AU [48] as implemented in IQ-TREE were used to test different scenarios of the placement of the yeast genes in the full dataset. In the best maximum likelihood scenario the yeast genes do not group as basal (Supplemental Fig. S1). Therefore, it was tested if a scenario where the yeast genes are basal in the full dataset is significant different from the best likelihood tree. A tree where yeast Sc-YAT1 and Sc-YAT2 group basal with CPT1s/CrAT/ChAT/CrOT and yeast Sc-CAT2 with the CPT2 family was also tested against the best likelihood tree as this scenario is in accordance with the expectation of the cellular placement of the genes.

2.3. Intron position analysis

Genomic sequences of the human, fruit fly and roundworm genes were compared to the corresponding cDNA and protein sequences in order to locate the intron positions within each gene. Yeast genes showed no introns and were therefore not included in the analyses. Positions less than 1 amino acid residue away from similar positions in orthologous and paralogous (e.g., CPT1A, B, C) counterparts were considered to be conserved. The genes were grouped (assigning a separate group for the extra CPT1-like genes in the roundworm) and the positions were plotted (Fig. 3). The alignments used were based on the core region, extended alignments for N-terminal (transit peptide) regions were applied when appropriate.

2.4. CPT1C evolution

The long branch between CPT1C of mammals and reptiles/birds suggest that positive selection could have occurred in the lineage leading to mammals. The dN/dS ratio of this branch was calculated to give more insight in the selection pattern, with dN being the number of nonsynonymous substitutions per nonsynonymous site, and dS the number of synonymous substitutions per synonymous site. To detect positive selection, the branch model, as implemented in PAML (v4.9a)

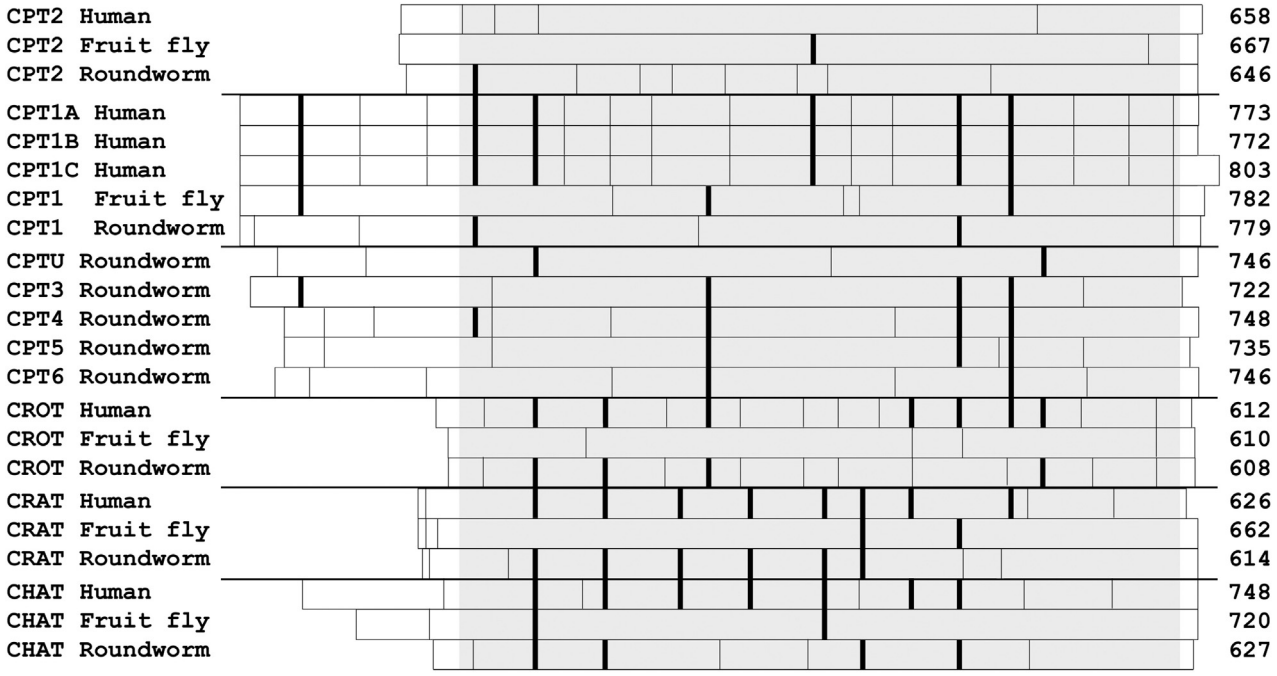

Fig. 3. Intron position conservation in carnitine/choline acyltransferase genes. The exon sequences of human-, fruit fly- and roundworm transferases are aligned as indicated (CPT1; roundworm CPT1-like proteins; CrOT; CrAT; ChAT and CPT2; number of amino acids residues on the right; only the coding parts of the exons are shown). The core region used for the alignment is indicated in light grey. The relative positions of introns are indicated by vertical bars. Fourteen conserved intron positions (conserved in more than one group) are highlighted by thick bars.

[49], was used to test for signs of positive selection in CPT1C. For robust estimation of positive selection an extended selection of CPT1Cs, meeting the criteria of full-length and error-free (see above), were extracted from Ensembl/NCBI databases (Supplemental Table S2). In these analysis the amphibians and reptiles/birds were used as outgroup. The estimated dS for the branch leading to the mammalian CPT1C was extremely high (54.8999), which suggests that the CPT1C divergence between reptiles/birds and mammals is too large to reliably calculate the dN/dS ratio for this branch.

3. Results

3.1. Phylogenetic analysis of the carnitine/choline acyltransferase family

A dataset consisting of 93 protein sequences of carnitine/choline acyltransferases family was retrieved from the Ensembl/NCBI databases (Supplemental Table S1) and used in different phylogenetic analyses. First, an initial phylogenetic tree was constructed including all the 93 protein sequences (Supplemental Fig. S1). This analysis firmly supports a grouping of yeast Sc-YAT1 and Sc-YAT2 with the CPT2 family, whereas yeast Sc-CAT2 strongly groups with the CrOT, CPT1s (A, B and C), CrAT and ChAT families. However, likely due to the deep evolutionary split of the two groupings, the yeast proteins are not basal as would be expected from their position compared to animals in the Tree of Life [50]. An evolutionary scenario placing the yeast proteins as basal was tested against the best maximum likelihood solution and could not be rejected (Table 1 and Supplemental Fig. S2). It was also tested if the yeast proteins could be swapped between the animal CPT2 and CrOT, CPT1s (A, B and C), CrAT and ChAT families as expected from the cellular placement of the proteins, but this scenario was significantly rejected (Table 1 and Supplemental Fig. S2).

The grouping of yeast Sc-YAT1 and Sc-YAT2 with the CPT2 family and yeast Sc-CAT2 with the CrOT, CPT1s, CrAT and ChAT family is also supported with the intron/exon structure of these genes of selected species (e.g. fruit fly, roundworm and human). Fig. 3 shows that several intron positions of the carnitine/choline acyltransferases genes remained conserved during evolution. Fourteen of these positions are still present in more than one group (highlighted in Fig. 3) and examples of these occur in each group. However, CPT2 has less conserved introns compared to the rest of the family. Based upon the result of the phylogenetic analysis and the conserved intron/exon positions, we therefore decided to perform separate phylogenetic analyses of the two groupings (Figs. 4 and 5).

The CPT2 family has developed from a common ancestor that is shared with yeast Sc-YAT1. The phylogenetic tree of the CPT2 family (Fig. 4) follows a regular topology and there are no signs of duplication events. The length of the branches towards the different species is relatively short, indicating that the protein sequences of CPT2, e.g. compared to CPT1, have remained relatively stable over time. From a common ancestor shared with yeast Sc-CAT2, four distinct families (CrOT, CrAT, ChAT and CPT1) have developed in the animal radiation, indicating that several duplications happened in the early evolution of animals (Fig. 5). Although not highly supported, the best fitting scenario is that the first duplication resulted in two clades that show distinctive acyl chain length preference (i.e. short chain versus medium- and long chain specificity). This is in accordance with the number of conserved intron position in these two clades; CrAT and ChAT have more conserved intron position compared to the other clade (CrOT and CPT1) (Fig. 3). The first clade shows a strong grouping of the families CrAT and ChAT indicating stronger conservation. Within this clade, the branch towards the ChAT family is longer compared to the branch towards CrAT, indicating that ChAT evolved with a higher evolutionary rate to become acetylcholine specific. CrAT remained the same in terms of specificity towards carnitine and acetylcarnitine and evolved slower. The other clade consists of the families CrOT and CPT1. In the CPT1 family, several duplications have taken place in the evolution of animals. We found five CPT1-like genes in *C. elegans*, which show all a strong grouping with the CPT1 family but branched from the root before the mammalian isoforms originated. In Fig. 6, the protein prediction including the transmembrane domain of the five CPT1-like genes of *C. elegans* are shown. It is highly supported (bootstrap value 100%) that vertebrate CPT1 isoforms group as a separate clade. Two duplications in

Table 1
Log likelihood values and test parameters for different evolutionary scenarios.

Model tested	logL	deltaL	bp-RELL	p-KH	p-SH	c-ELW	p-AU
H0. Tree A	−54,235.652	0	0.3919+	0.4883+	0.8051+	0.4033+	0.6065+
H1. Tree B	−54,241.972	6.32	0.2017+	0.2106+	0.3846+	0.1868+	0.2442+
H2. Tree C	−54,257.491	21.839	0.0059−	0.0407−	0.0412−	0.0066−	0.0135−

Notes: Tree A = tree with best maximum likelihood; Tree B = Yeast Sc-CAT2 basal to CPT1s/CrAT/ChAT/CrOT family; Tree C = Yeast Sc-YAT1 and Sc-YAT2 basal with CPT1s/CrAT/ChAT/CrOT family and Yeast Sc-CAT2 basal with the CPT2 family; deltaL = logL difference from the maximal logL in the set; bp-RELL = bootstrap proportion using RELL method [44]; p-KH = p-value of one sided Kishino-Hasegawa test [45]; p-SH = p-value of Shimodaira-Hasegawa test [46]; c-ELW = Expected Likelihood Weight [47]; p-AU = p-value of approximately unbiased (AU) test [48].

this clade resulted in the vertebrate isoforms A, B and C. Since bootstrap values are low for these duplications the order of appearance remains unsolved (illustrated by different outcomes shown in Fig. 5, and Supplemental Figs. S1, S4, S6 and S7). The results obtained by maximum likelihood analysis of the CPT2 and CPT1 phylogeny were confirmed using a Bayesian analysis method (i.e., Mr. Bayes [42]) (Supplemental Figs. S4–S7).

Together with the molecular phylogenetic analysis, the intron/exon position of these genes confirms that all these genes evolved from one common ancestor and that a eukaryotic “intron early” hypothesis can be applied to this family [52]. Moreover, a retroposition event as basis for the duplication of the CPT2 ancestor can likely be ruled out, since different introns appear at conserved positions in the non-human orthologues (Fig. 3).

3.2. CPT1C

The tree topology shows that the branch towards the CPT1C family is much longer compared to the branch towards CPT1A and CPT1B, indicating that, compared to their common ancestor, the structure of CPT1C has changed much more than CPT1A and CPT1B (Fig. 5). A molecular phylogenetic analysis of all CPT1 genes has been performed to study the evolution of CPT1C in more detail. In Fig. 7 it is shown that CPT1C is present in lobe-finned fish, ray-finned fish, amphibia, reptiles, birds and mammals. The branch leading to mammalian CPT1C genes is much longer than the branches between other CPT1C species and the branches leading mammals in CPT1A and CPT1B. Additional analyses focused on the contribution of different driving forces behind the changes of mammalian CPT1C. One possibility would be that strong positive selection may have occurred in this lineage. To investigate this, branch model [49] was used to detect positive selection. However, the dS of this branch could not be calculated reliably, as we obtained an unrealistic high dS rate of 54.8999. Thus the sequences are probably too distinct for this kind of analysis, but the long branch does suggest that a high degree of positive evolution has happened in this branch.

4. Discussion

4.1. Co-evolution of CPT2 and CPT1

The enzymes of the carnitine/choline acyltransferase family play very diverse but specific roles. Therefore, it is not surprising that each group of these transferases has been well conserved during evolution. We particularly show (a) an early separation of CPT2 on the one hand and CrOT, CPT1s (A, B and C), CrAT and ChAT on the other, (b) that ChAT seems to be evolved from CrAT, gaining its choline specific

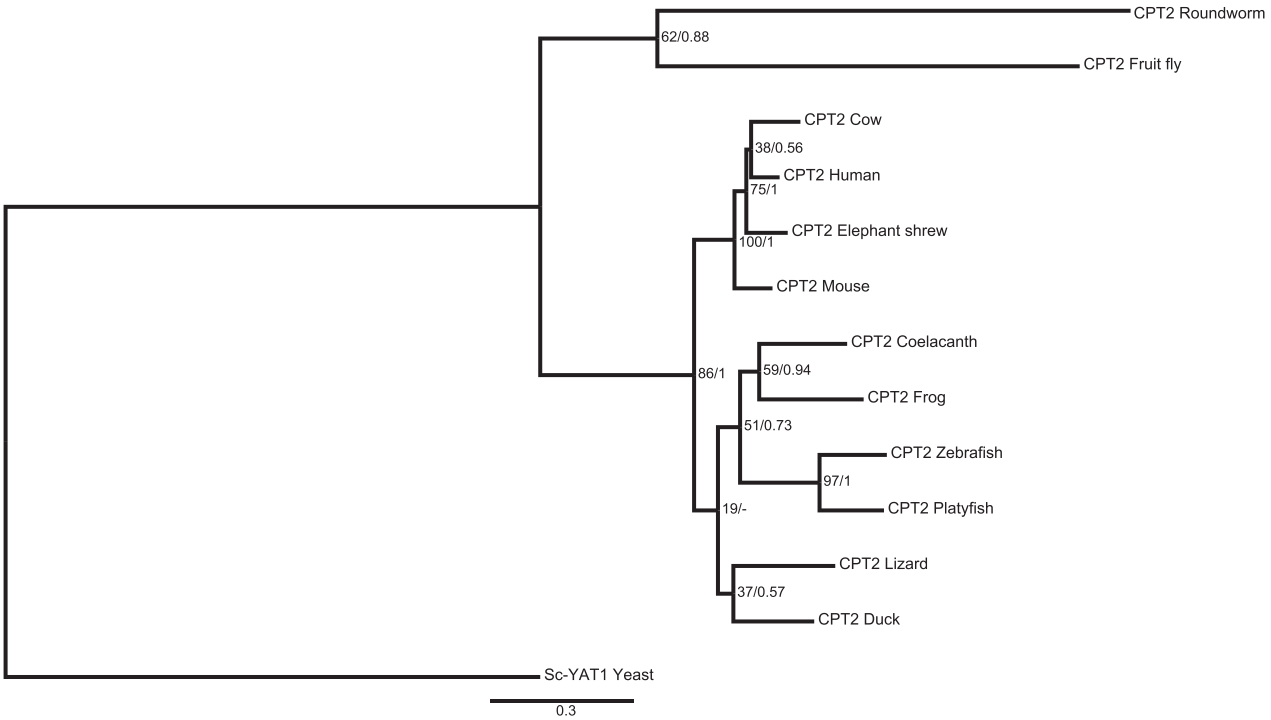

Fig. 4. Phylogenetic tree of the CPT2's using Sc-YAT1 of *Saccharomyces cerevisiae* as outgroup. Branch lengths are equal to the amino acid changes. The node values represent bootstrap support values and posterior probability from maximum likelihood and Bayesian analysis, respectively. The symbol “-” indicates that this node is not supported in Bayesian analysis. The Bayesian Phylogenetic tree is in Supplemental Fig. S5.

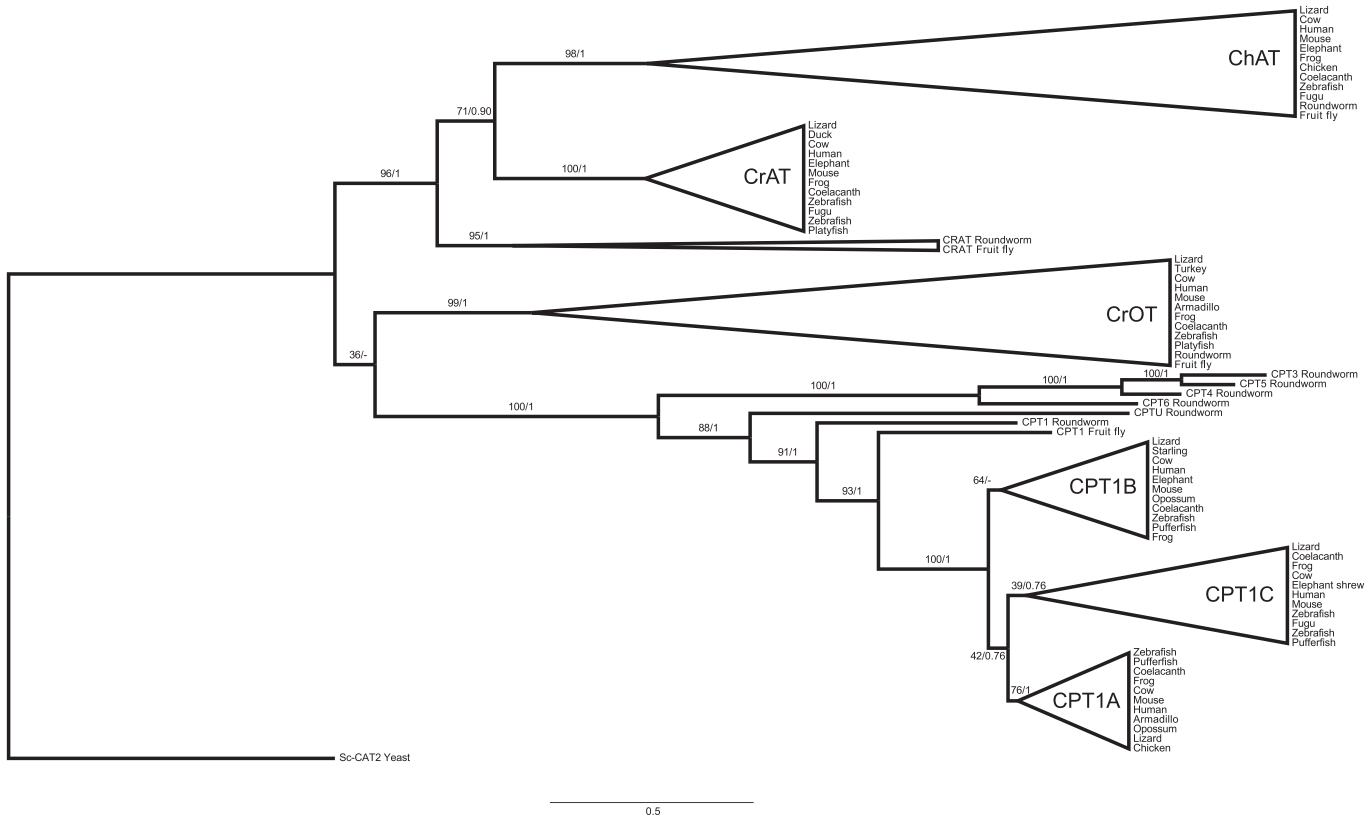

Fig. 5. Phylogenetic tree of the carnitine and choline acyltransferases CrOT, CrAT, ChAT and CPT1 using Sc-CAT2 of *Saccharomyces cerevisiae* as outgroup. Branch lengths are equal to the amino acid changes. The node values represent bootstrap support values and posterior probability from maximum likelihood and Bayesian analysis, respectively. The symbol "-" indicates that this node is not supported in Bayesian analysis. For better visualisation, the nodes of the different families have been collapsed. The bootstrap value of 44 at the node of the non-CrOT genes is low and allows other tree topologies (Supplemental Figs. S1 and S2). Full topology of this tree is in Supplemental Fig. S3. The Bayesian phylogenetic tree is in Supplemental Fig. S6.

function, (c) the existence of five CPT1-like genes next to a "true" CPT1 in *C. elegans* and (d) a striking long evolutionary branch towards mammalian CPT1C.

Intron position conservation of the carnitine/choline acyltransferase family strongly suggests a common evolutionary ancestry. Sc-YAT1 and Sc-YAT2 are more closely related to CPT2 than to the other acyltransferases, whereas Sc-CAT2 is more closely related to the other acyltransferases, including CPT1. A common ancestor evolved into Sc-YAT/CPT2 on the one hand and into Sc-CAT2/CPT1 on the other. Although the yeast genes are not basal in the full data analysis (Supplemental Fig. S1), a tree with the yeast genes basal is not significantly different from the best likelihood tree (Table 1 and Supplemental Fig. S2). The deviation may be explained by a phenomenon called "long branch attraction". On the other hand, a tree with the yeast genes being swapped between the two animal CPT families is rejected.

While duplications occurred in the CPT1 family, this is not the case for the CPT2 family. Although CPT2 is essential [52], it is expressed constitutively, is not regulated and not rate-limiting. This is in contrast to the members of the CPT1 family, which may explain the absence of duplications in the CPT2 family.

It is remarkable that an exchange in subcellular location of the mitochondrial enzymes CPT2 and CPT1 seems to have occurred compared to the location of the closest relatives of their ancestors. Ancestral CPT2 representatives Sc-YAT1 and Sc-YAT2 are located at the mitochondrial outer membrane and in the cytosol respectively [25,26], whereas CPT2 itself is located in the mitochondrial matrix where it interacts with the inner membrane. Furthermore, the ancestral CPT1 representative Sc-CAT2 is located in the mitochondrial matrix, whereas mammalian CPT1 is located in the outer membrane [24]. This may indicate that during evolution the animal proteins CPT1 and CPT2 switched their location from the mitochondrial matrix to the mitochondrial outer membrane and vice versa. It is tempting to speculate that the observed switch in subcellular location is the result of alterations in mitochondrial localization signals, possibly associated with evolutionary development of the mitochondrial protein import machinery, resulting in an alternative routing (outer membrane vs matrix) during mitochondrial protein import [53]. Alternatively, the carnitine/choline acyl transferase family may have originated from an ancient endogenous mitochondrial encoded protein, which would thus not require a transit peptide as a localization signal. During subsequent evolution of the different branches of transferases, specific N-terminal extensions (i.e. transit peptides or N-terminal membrane anchoring sequences) would allow proper localization of the proteins. This scenario agrees with the differences between the transit peptides of Sc-CAT2, CrAT and CPT2 that we noted previously [5]. Although the transit peptides are too short to allow firm conclusions with respect to conservation, the notion is supported by the specific localization signals of CPT1 that are different from intramitochondrial proteins [54,55].

4.2. ChAT

Considering the non-CPT2 related acyltransferases, ChAT is the only member in this family that transfers acetyl-CoAs to choline instead of carnitine. As Fig. 5 shows, ChAT is most closely related to CrAT. The long evolutionary branch towards ChAT indicates that ChAT has evolved beyond CrAT to obtain its choline specific function. This scenario is supported by site-directed mutagenesis experiments, that show that only four amino acid substitutions suffice to provide ChAT with specificity for choline, rather than for carnitine [56]. Although it was known that choline and carnitine acetyltransferases are evolutionary

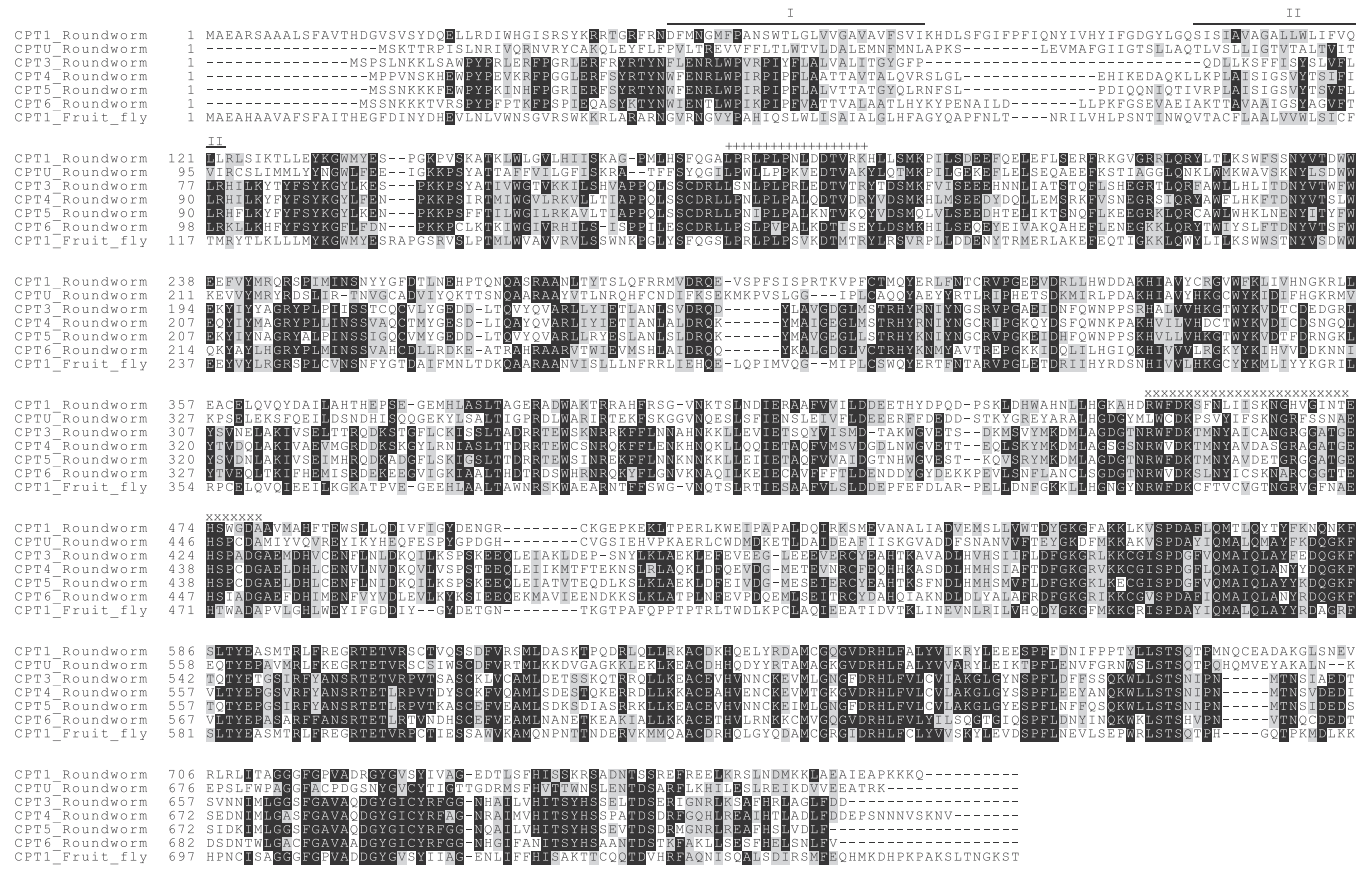

Fig. 6. Protein predictions and amino acid alignment of the "true" CPT1 and five CPT1-like genes of *C. elegans*, and CPT1 of *Drosophila melanogaster*. The alignment is shown with shaded residues at positions where these are identical (black) or similar (grey). The two CPT1 transmembrane (TM) domains are indicated with roman numbered bars above the sequence alignment. Carnitine acyltransferase motifs are indicated by + symbols (Prosite PS00439) and X symbols (Prosite PS00440). As shown, all the CPT1-like genes of *C. elegans* contain the acyltransferase motifs as well as the two TM domains, albeit that CPT3 seems to lack a loop in between the TM domains.

connected, the position of the ChAT branch has now been established.

4.3. CPT1 genes in Caenorhabditis elegans

In our database searches and subsequent phylogenetic analyses we found evidence for the existence of several CPT1-like genes in *C. elegans*. Next to one "true" CPT1, that approximately is of the same length as mammalian CPT1A, CPT1B and CPT1C and fruit fly CPT1 (Fig. 3), there are 5 additional CPT1-like genes (denoted CPTU and CPT3-CPT6) that arose from duplications earlier in evolution. These seem to be actively expressed and the encoded proteins all suit the common characteristics of CPT1 protein structure, albeit with a slightly shorter N-terminal region containing the transmembrane domains (Fig. 6). Moreover, CPT3 seems to lack a loop in between the transmembrane domains. To our knowledge, no experimental data on the enzyme characteristics and kinetics of these proteins is known. However, deviant or absent malonyl-CoA sensitivity can be anticipated given the absence of key residues known to interact with the C-terminal regulatory site in mammalian CPT1A and CPT1B [13]. Further study of the biochemical and physiological functions of the additional CPT1-like genes is warranted, also because of variation in subcellular location of CPT1 isoforms in mammals (see Section 4.4).

4.4. CPT1C

In the evolution of CPT1, several duplications have occurred, and in mammals and other vertebrates these have led to the isoforms CPT1A, CPT1B and CPT1C. Lopes-Marques et al. also studied the origin and evolution of CPT1 genes [17]. In contrast to earlier findings, they found that CPT1C not only is present in teleosts, but also in amphibia, reptiles and lobe-finned fish. In our study we confirm their analysis. Lopes-Marques et al. also showed a long branch in the CPT1C mammalian lineage, indicative of a high evolutionary rate, but did not provide an explanation. To interpret the remarkable evolutionary history of CPT1C, we tried to estimate patterns of evolutionary selections (purifying, relaxed/positive) but the divergence of the reptile/bird and mammalian CTP1Cs is too large to reliably estimate these evolutionary forces. However, the long branch does suggest that a high degree of positive and/or relaxing evolution must have occurred.

Although our calculations of dN/dS ratios failed to yield reliable results when mammalian CPT1C genes were compared to orthologues in lizards and birds, we noted large differences in GC content at the third positions of codons of CPT1A as well as CPT1C (data not shown). These so called GC3 values are very different between human codons and those of e.g., the lizard *Anolis carolinensis* [57] and contribute to errors in calculation of dN/dS. The reason for these GC3 differences remains obscure. Tissue specific tRNA sets and consequential codon adaptation has been suggested as an explanation [58], but evidence for this is poor [59].

A physiological explanation for the long CPT1C branch may be its involvement in the regulation of food intake [15,60]. CPT1 enzymes are inhibited by malonyl-CoA [11,13,61], and synthesis of malonyl-CoA by acetyl-CoA carboxylases is under the control of AMP-activated protein kinase (AMPK) [11,62]. AMPK is a cellular energy sensor and regulator of energy homeostasis [63]. In the brain, AMPK also regulates whole body energy homeostasis by regulating feeding behaviour and energy

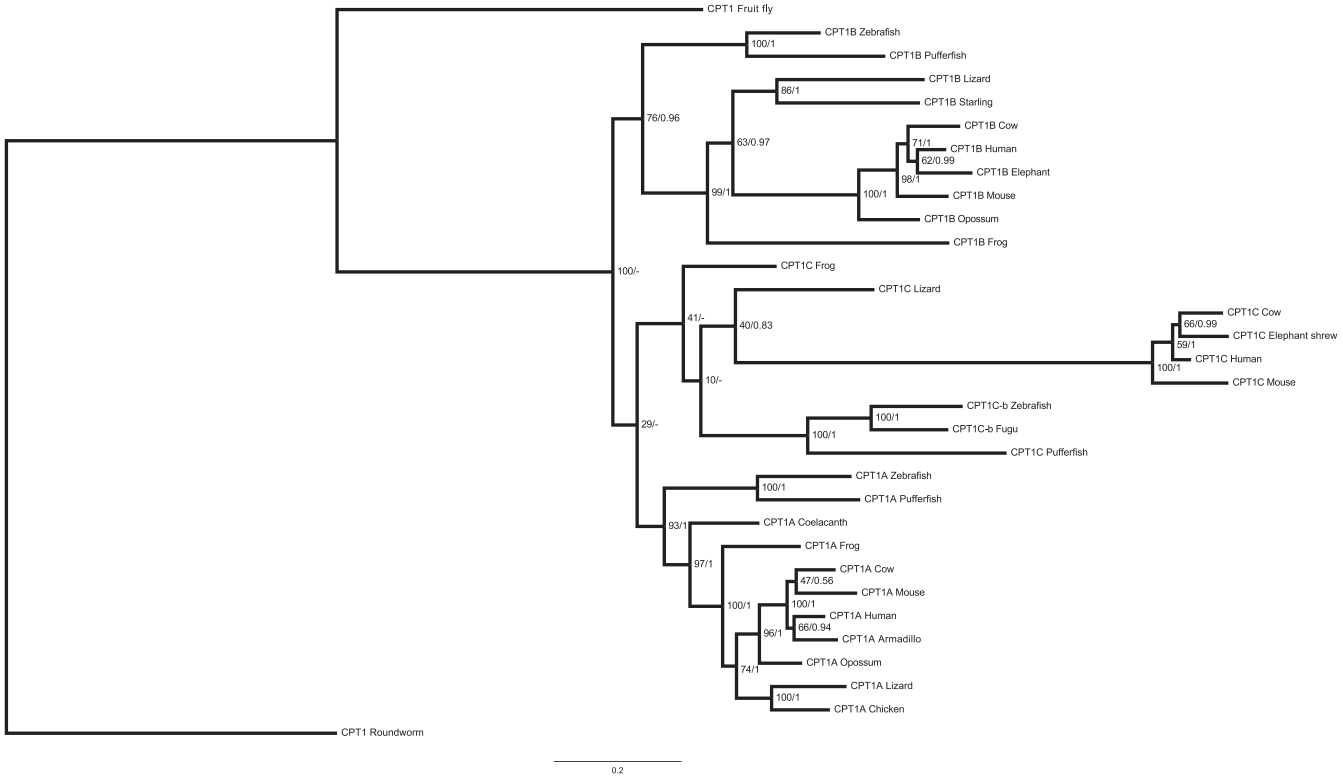

Fig. 7. Phylogenetic tree of CPT1A, CPT1B, and CPT1C. Branch lengths are equal to the amino acid changes. The node values represent bootstrap support values and posterior probability from maximum likelihood and Bayesian analysis, respectively. The symbol "-" indicates that this node is not supported in the Bayesian analysis. It can be inferred that the branch towards mammalian CPT1C is long compared to the other branches in this tree. The long branch indicates that several amino acids have been changed and that positive evolution might have happened. The Bayesian phylogenetic tree is in Supplemental Fig. S7.

expenditure [64], in which CPT1C is implicated. This notion is supported by experimental brain-specific CPT1C gene inactivation, which results in a decreased food intake and a lower body weight in mice with inactive CPT1C compared to their wildtype littermates [65]. Both function and subcellular location (Fig. 1) of mammalian CPT1C markedly differ from CPT1A and CPT1B, and this may relate to its long branching.

5. Conclusions

To conclude, although CPT2 and CPT1 must have co-evolved to establish the carnitine shuttling, these enzymes are the most distantly related carnitine transferases, split early in evolution, during which a switch occurred in their location, from the mitochondrial matrix to the mitochondrial outer membrane and vice versa. ChAT is most closely related to CrAT and has evolved to a further extend than CrAT to gain its choline specific function. CPT1 has duplicated several times during evolution, resulting in the isoforms CPT1A, CPT1B and CPT1C, and, in *C. elegans*, in five extra CPT1-like genes. CPT1C is the brain specific isoform that resulted from positive and/or relaxed selection in the mammalian lineage. Our evolutionary delineation of the mammalian carnitine/choline acyltransferases fits current knowledge on their functions. It extends this knowledge and provides tangible leads for further experimental research into the functioning of this fascinating enzyme family with a key role in metabolism and signalling.

Author contributions

FL and JK designed the research. OM performed the data analysis with help of MH. MH wrote the manuscript, except for the method section which was written by OM. MH prepared most of the figures. FL, JK and OM edited the manuscript.

Disclosure statement

The authors declare no conflicts of interest associated with this manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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