ONTOGENIC EXPRESSION AND CHARACTERIZATION OF THE LONG-CHAINED POLYUNSATURATED FATTY ACID BIOSYNTHESIS ENZYME, ELONGASE IN ZEBRAFISH

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LIST OF SYMBOLS

nl	nanoliter
µl	microliter
ml	milliliter
ng	nanogram
µg	microgram
mM	mili molar
n-3	Omega 3
n-6	Omega 6
Δ5	Delta 5
Δ6	Delta 6
Δ9	Delta 9

LIST OF ABBREVIATIONS

DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
hpf	hour post fertilization
LB	Luria Bertani
MAB	Maleic Acid Buffer
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RNA	Ribonucleic Acid
RNasin	Ribonuclease inhibitor
RT-PCR	Real Time Polymerase Chain Reaction
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
NBT	Nitroblue tetrazolium
HYB^-	Hybridization buffer without torula (yeast) RNA and heparin
HYB^+	Hybridization buffer with torula (yeast) RNA and heparin
kDa	kilodalton
MO	Morpholino
GFP	green fluorescent protein
dH_2O	distilled water
NaCl	Sodium chloride
$MgCl_2$	Potassium chloride
HCl	Hydrochloride acid
DMF	Dimethylformamide
SDS	Sodium dodecyl sulphate
PAGE	PolyAcrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
APS	Ammonium persulphate
mRNA	messenger RNA
PVDF	Polyvinylidene fluoride

PENGEKSPRESAN DAN PENCIRIAN ONTOGENIK ENZIM SINTESIS ASID LEMAK PANJANG POLI-TAK-TEPU, ELONGASE DALAM IKAN ZEBRAFISH

ABSTRAK

Kepentingan asik lemak rantai panjang poli-tak-tepu (LC-PUFAs) dalam perkembangan vertebrata telah dikaji secara luasnya. Antaranya, ia memainkan peranan dalam pengekalan integriti membran sel, pengisyaratan sel, pengawalaturan gen dan metabolisme. LC-PUFAs seumpama asik arakidonat, asik eikosapentaenoat dan asik dokosaheksaenoat juga mempengaruhi proses fisiologi seperti keradangan, kekebalan, pembiakan dan perkembangan. Walaupun, kepentingan LC-PUFAs dalam perkembangan telah diketahui, hanya terdapat sedikit pengetahuan tentang pemanfaatan dan biosintesisnya semasa embriogenesis. Embrio zebrafish memiliki kelebihan unggul sebagai model organisma dalam biologi perkembangan, ini termasuklah kemudahan visualisasi, ketersediaan jumlah anak yang banyak dan kewujudan kaedah dan peralatan molekul. LC-PUFAs disintesis melalui langkah-langkah penyahtepuan dan pemanjangan berganti dengan enzim utama: desaturase (enzim penyahtepuan) dan elongase (enzim pemanjangan). Kajian ini menerangkan pengekspresan dan pencirian fungsi dua ahli elongase, *elovl5* dan *elovl2* dalam embriogenesis zebrafish. *elovl5* diekspres pada tahap tertinggi yang signifikan pada peringkat awal embrio menandakan proses pewarisan gen daripada ibu. Sebaliknya, *elovl2* diekspres pada tahap yang rendah pada peringkat awal embrio dan semakin meningkat ke tahap tertinggi pada peringkat akhir embrio. *elovl5* dan *elovl2* didapati diekspres dalam otak pada seawal-awalnya 24 jam lepas persenyawaan. Namun, semasa embrio membesar, gen tersebut memaparkan pengekspresan pada lokasi yang berbeza. *elovl5* secara khususnya diekspres di tubul ginjal dan lapisan sinsitium

kuning telur sementara *elovl2* diekspres di hati dan usus. Sebagai tambahan, analisis asik lemak menunjukkan penurunan keseluruhan substrat dan peningkatan produk LC-PUFAs semasa embrio zebrafish berkembang, berpadanan dengan peningkatan tahap pengekspresan kedua-dua elongase tersebut. Pencirian fungsi kedua-dua gen tersebut dikaji dengan menggunakan pendekatan penindasan gen. Penindasan gen *elovl5* dan *elovl2* secara berasingan, menghasilkan mikrosefali (otak bersaiz kecil) pada peringkat awal embrio dan akhirnya busung perikardium dan pundi kuning telur dihasilkan. Dalam penindasan gen *elovl5*, seolah-olah tiada kesan atas perkembangan ginjal kerana segmentasi tubul ginjal yang sempurna dibuktikan melalui penghibridan *in situ* dengan penanda yang berpadanan. Analisis penapisan pewarna dan pengambilan semula menunjukkan tubul ginjal berfungsi dalam morfan (embrio yang ditindas gen). Penemuan seperti ini menimbulkan spekulasi bahawa busung yang terhasil disebabkan gangguan sekatan kulit tetapi bukannya ginjal. Dengan menggunakan D-mannitol untuk meningkatkan keosmolaran air sekeliling morfan *elovl5* atau *elovl2*, maka dapat disahkan bahawa penindasan gen *elovl5* dan *elovl2* mengganggu sekatan ketelapan air dan seterusnya menghasilkan embrio berbusung. Tambahan, profil asik lemak menunjukkan penurunan asik arakidonat dalam kedua-dua morfan penindasan *elovl5* dan *elovl2* menimbulkan spekulasi bahawa asik arakidonat adalah penting untuk mengekalkan integriti sekatan ketelapan air kulit.

ONTOGENIC EXPRESSION AND CHARACTERIZATION OF THE LONG-CHAINED POLYUNSATURATED FATTY ACID BIOSYNTHESIS ENZYME, ELONGASE IN ZEBRAFISH

ABSTRACT

The importance of long-chained polyunsaturated fatty acids (LC-PUFAs) in vertebrates' development has been widely studied. Among these, include roles in maintaining cellular membrane integrity, cellular signaling, gene regulation and metabolism. LC-PUFAs such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid also mediate physiological processes like inflammation, immunity, reproduction and development. Despite the known importance of LC-PUFAs during development, very little is known about their utilization and biosynthesis during embryogenesis. Zebrafish embryos possess distinct advantages as model organism in developmental biology, including the ease of visualization, the availability of large numbers of offspring coupled with the existing molecular methods and tools. LC-PUFAs are synthesized via alternating desaturation and elongation steps with the key enzymes: desaturase and elongase. This present study describes the expression and functional characterization of two family members of elongases, *elovl5* and *elovl2* in zebrafish embryogenesis. *elovl5* is significantly expressed at highest level at initial embryogenic stage which imply the maternal transfer process. Conversely, *elovl2* is expressed at low level at initial stage and gradually elevated as embryo developed. Spatially, both *elovl5* and *elovl2* are found expressed in brain as early as 24hpf. However, as the embryos develop, they displayed distinct spatial expression. e*lovl5* is specifically expressed in pronephric tubules and yolk syncytial layer while *elovl2* predominantly expressed in liver and intestine. In addition, profiling of fatty acids shows a general decrement of the

substrates and increase of the LC-PUFAs as the embryo develops, which match the increasing expression of both elongases. Functional characterization of both genes was elucidated using knockdown approach. Knockdown of *elovl5* and *elovl2*, respectively produced microcephaly embryos at early embryonic stage and eventually pericardial and yolk sac edema developed. In *elovl5* knockdown, there seems to be no effect on pronephric development as intact segmentation of the pronephric tubules in morphants was confirmed by *in situ* hybridization using corresponding markers. Dye filtration and reuptake analysis also showed functional pronephric tubule in morphants. These findings raised the speculation that the edema comes from disrupted skin barrier instead of kidney. By using D-mannitol to increase the osmolarity of the water surrounding either *elovl5* or *elovl2* morphants, we confirmed that *elovl5* and *elovl2* perturbation disrupts the water permeability barrier eventually resulting in edematous embryos. In addition, fatty acid profile showed decrement of arachidonic acid in both *elovl5* and *elovl2* knockdown morphants led to the speculation that arachidonic acid is crucial in maintaining skin integrity.

CHAPTER 1

INTRODUCTION

1.1 Polyunsaturated fatty acids (PUFAs) and long-chained PUFAs (LC-PUFAs)

Polyunsaturated fatty acids (PUFAs) are fatty acids with more than one carbon-carbon double bonds within the carbon chain. PUFAs were first described in late 1920s and early 1930s in the reports on dietary fats that contained two vitamin-like substances identified as linoleate (18:2n-6) and α-linoleante (18:3n-3) (Cunnane, 2003). Initially, these two "parent" PUFAs were jointly known as vitamin F and later termed as essential fatty acids (EFA).

Long-chained polyunsaturated fatty acids (LC-PUFAs) are fatty acids of more than 18 carbons chain, containing more than one double bond within the chain. LC-PUFAs that have been widely documented are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA). LC-PUFAs may be of dietary or cellular origin. The importance and beneficial effects of LC-PUFAs have long been recognized. In terms of biological processes, LC-PUFAs are the major constituent of membrane conferring the fluidity, flexibility and selective permeability to cellular membranes (Wallis *et al.*, 2002; Stillwell and Wassall, 2003). In addition, LC-PUFAs in membrane phospholipid also serve as metabolic precursors of signaling molecule such as eicosanoids and docosanoids that modulate intercellular communication and autocrine signaling from the plasma membrane (SanGiovanni and Chew, 2005; Serhan, 2005; Kirkup *et al.*, 2010). LC-PUFAs such as ARA and EPA are precursors of eicosanoids, producing prostaglandins, leukotrienes and thromboxanes. These eicasanoids are key regulators of cell proliferation,

differentiation, oxidative stress and neuroinflammation. On the other hand, DHA can be converted to docosanoids, including docosatrienes, neuroprotectins and resolvins, which produce antioxidant, anti-inflammatory and anti-apoptotic effects. Nutritional alteration which led to qualitative and quantitative changes in cellular responses to extracellular signals suggested that fatty acids can directly and indirectly modulate signaling pathways at multiple levels (Hwang and Rhee, 1999). Apart from these, LC-PUFAs also act as regulators of gene expression and specifically cause changes in cellular metabolism, differentiation and growth (Sampath and Ntambi, 2004; Sampath and Ntambi, 2005). Specifically, PUFAs are ligands to several transcription factors and affect gene expression through regulation of corresponding transcription factors such as PPAR, RXR and SREBP.

LC-PUFAs also mediate numerous physiological processes such as inflammatory (Simopoulos, 2002; Calder, 2006), cognitive and neurodevelopment (Willatts and Forsyth, 2000; Wainwright, 2002; Innis, 2007; Farooqui, 2009), retinal development (Martin *et al.*, 1994; SanGiovanni and Chew, 2005; Schnebelen *et al.*, 2009) and cardiovascular diseases (Calder, 2004; Breslow, 2006).

1.1.1 LC-PUFAs and their biosynthetic pathway

The biosynthesis of LC-PUFAs in vertebrates involve serial desaturation and elongation that convert the C18 precursors, α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) to longer and more unsaturated fatty acids of the same series. n-3 series or omega-3 (ω-3) PUFAs is a family of polyunsaturated fatty acids with their first carbon-carbon double bond located at the third carbon from the methyl terminal (CH_3). The same nomenclature system is applied to the n-6 series or omega-6 (ω-6) PUFAs which have the first carbon-carbon double bond in the sixth carbon from the methyl end (Davidson and Cantrill, 1985).

EPA and DHA are n-3 metabolites from α-linolenic acid whereas ARA is an n-6 metabolite converted from linoleic acid. n-3 and n-6 LC-PUFAs are synthesized through two separate pathways involving sequential desaturation and elongation. Two key enzymes, desaturase and elongase have been identified functioning in concert in these pathways. Desaturases are responsible in adding the double bond to the fatty acid chain whereas the elongases are involved in chain elongation by adding carbon atom (details of elongases described in section 1.2). To date, a few subtypes of mammalian desaturases have been identified: Δ9 also referred as *SCD* was the first desaturase isolated (Strittmatter *et al.*, 1974) followed by Δ5 fatty acyl desaturases (*fad*) (Cho *et al.*, 1999a) , Δ*6 fad* (Aki *et al.*, 1999; Cho *et al.*, 1999b) and the recently isolated Δ*4 fad* (Li *et al*.). Amongst, Δ5 and Δ6 fatty acyl desaturases are critical enzymes in LC-PUFAs synthesis that function in the first step of LC-PUFAs conversion.

In 1990s, biosynthesis pathways of LC-PUFAs were established in rat (Voss *et al.*, 1991) and rainbow trout (Buzzi *et al.*, 1996; Buzzi *et al.*, 1997). The pathways appeared similar in rat and rainbow trout, at least qualitively (Tocher, 2003). Hagve *et al.* (1986) also found similarity of chain elongation in liver cells isolated from rat and rainbow trout. The biosynthetic pathways that have been established in other species have appeared similar. The general n-3 and n-6 biosynthetic pathways for primary PUFAs and their metabolites with corresponding enzyme catalysts are presented in Figure 1.1.

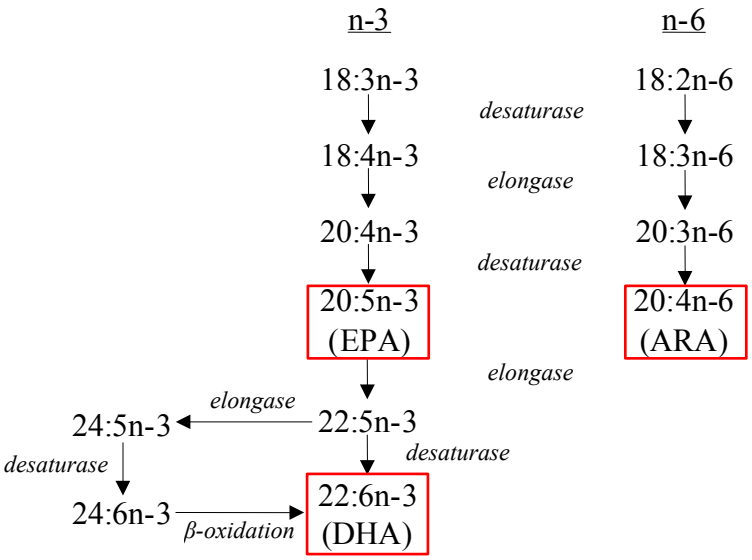

Figure 1.1 Biosynthetic pathways of the n-3 and n-6 LC-PUFAs from their precursors. LC-PUFAs are synthesized through series of desaturation and elongation catalysed by desaturase and elongase. n-3 and n-6 LC-PUFAs that commonly studied are framed.

1.2 Elongases

Elongase, elongase system, or fatty acid chain elongation system (FACES) all refer to enzymes that are responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain. Cellularly, fatty acid elongation occurs in three cellular compartments: cytosol, mitochondria and endoplasmic reticulum (ER) (Armstrong and Jump, 2009). The occurrence of fatty acid chain elongation in the endoplasmic reticulum was first discovered by Nugteren (1965). In his pioneering work, he found that the chain elongation enzymes present mainly in the microsomes and four serial reactions were found occuring in the chain elongation mechanism. Each step discussed in detail by Cinti *et al.* (1992), which the first step is condensation of acyl-CoA and malonyl-CoA resulting in β-ketoacyl-CoA. The second step is a reduction reaction where the β-ketoacyl-CoA is converted to β-hydroxyacyl-CoA utilizing NAPDH. β-hydroxyacyl-CoA is subsequently dehydrated

in the third step. The dehydration step resulted in enoyl-CoA which needs to be reduced by enoyl-reductase in the forth step to complete the elongation cycle. Nugteren (1965) postulated that the first reaction is the rate-limiting step based on the rates obtained for the four individual reactions. Later, Bernert and Sprecher (1979) and Cinti *et al.* (1992) also provided unequivocal proof that the rate of condensation is identical with total elongation and suggested that the condensing enzyme determines substrate specificity.

The malonyl-CoA-independent fatty acid synthesis system in mitochondria is only capable of elongating fatty acids (Harlan and Wakil, 1962; Barron, 1966; Christ, 1968; Charles F. Howard, 1970; Howard, 1970). Mitochondrial elongation system is distinctive from chain elongation in ER and had been elucidated by Seubert and Podack (1973). Fatty acid synthesis is elongated by the addition of acetyl-CoA independent from malonyl-CoA, which primarily elongates shorter fatty acid (< C16). Through kinetics studies, enocyl-CoA reductase was found as the rate-limiting enzyme. However, the ER system predominates quantitatively.

Later, extensive biochemical studies of the fatty acid elongation machinery suggested the existence of several distinct elongation pathways before genomic sequences for the enzyme were available (Sprecher, 1974). Numerous studies suggested the existence of multiple condensing enzymes for saturated, monounsaturated and polyunsaturated acyl-CoA in fatty acid elongation (Prasad *et al.*, 1986; Suneja *et al.*, 1990; Suneja *et al.*, 1991). Genes required for elongation of fatty acyl-CoA were first isolated from the yeast, *Saccharomyces cerevisiae* which resulted in identification of three separate elongase genes, designated ELO1, ELO2 and ELO3 (Toke and Martin, 1996; Oh *et al.*, 1997). Such genes were later identified in plants and mammals which have been reviewed in detail by Leonard *et al.*(2004).

The elongases were designated Elovl for elongation of very long fatty acids. To date, there are seven elongase subtypes (Elovl1-7) have been identified in mouse, rat and human. Based on their conserved motifs in their corresponding protein sequences, such as KXXEXXDT, HXXHH, HXXMYXYY, TXXQXXQ (Leonard *et al.*, 2004; Meyer *et al.*, 2004) they are grouped as a gene family, which are refered as members of elongase gene (Figure 1.2 and Figure 1.3).

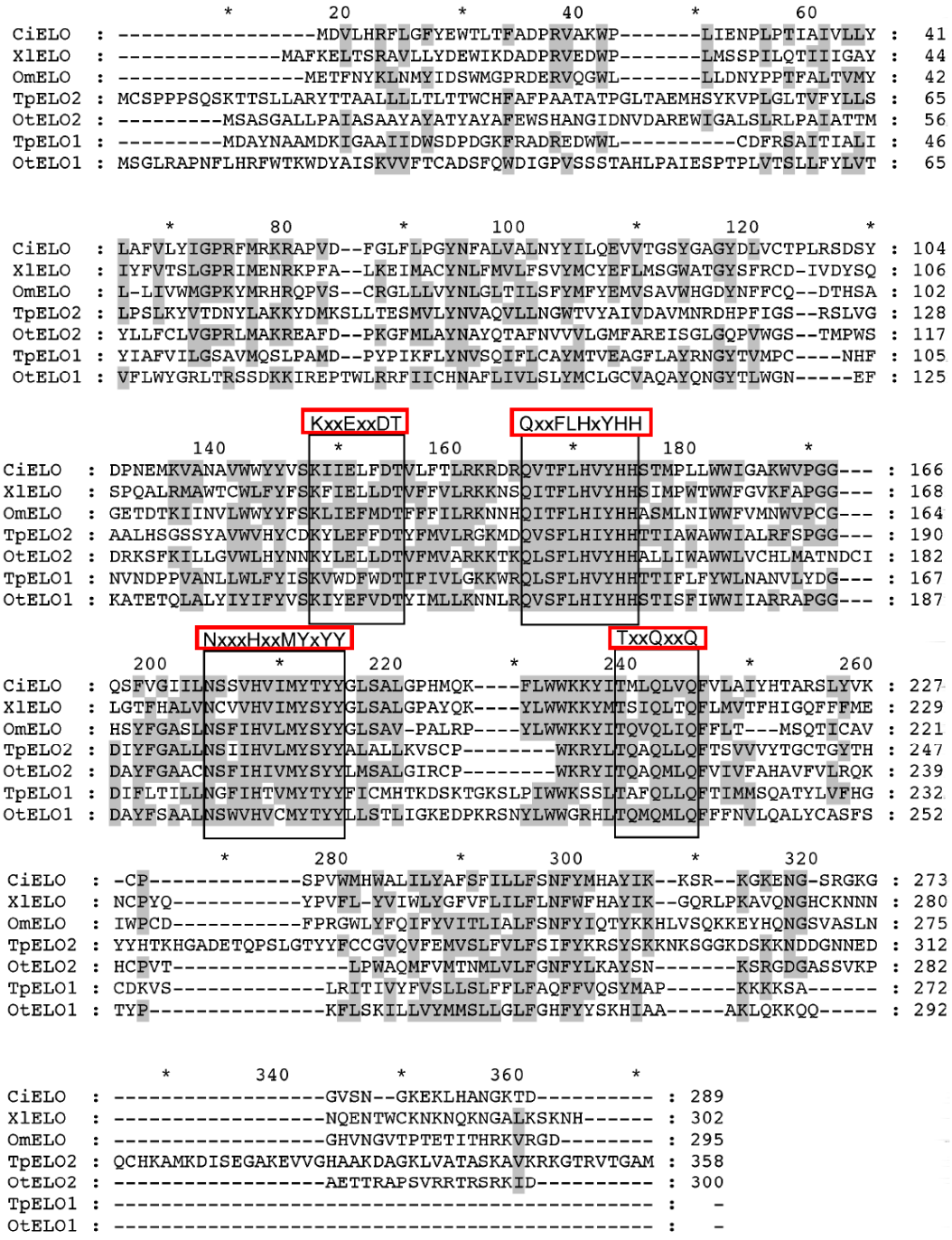

Figure 1.2 Amino acid sequences of elongases deduced from various species showing the conserved motifs which are framed.

Key: CiELO: *Ciona intestinalis* (sea vase); XlELO: *Xenopus laevis* (frog); OmELO: *Oncorhynchus mykiss* (rainbow trout); TpELO1 and TpELO2: *Thalassiosira pseudonana* (marine diatom); OtELO1 and OtELO2: *Ostreococcus tauri* (marine green alga). Amino acid alignment modified from Meyer *et al.* (2004).

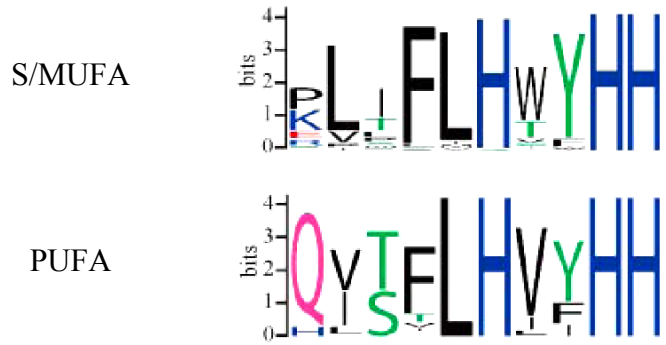

Figure 1.3 Sequence logo shows conserved histidine box between elongase subfamilies. Several amino acids are different at the N-terminal side of the histidine box, such as lysine in the S/MUFA subfamily and glutamine in the PUFA subfamily. The height of each amino acid symbol is proportional to its frequency of occurrence.

Key: S/MUFA: saturated or monounsaturated faty acid; PUFA: polyunsaturated fatty acid. Figure is adapted from Hashimoto *et al.* (2008).

Functionally, elongases diverge into two phases based on the range of substrate specificities. In the first phase, elongases deviate into two subfamilies depending on the substrate specificities: S/MUFA elongase (elongating saturated or monounsaturated fatty acids) and PUFA elongase (elongating polyunsaturated fatty acids) (Meyer *et al.*, 2004). These subfamilies can be distinguished based on sequence similarities, because different amino acids are conserved. In the second phase, a variety of substrate specificities diverged within the individual subfamilies (Hashimoto *et al.*, 2008). *Elovl1*, *elovl3*, *elovl6* and *elovl7* which are S/MUFA elongases, show preference towards saturated and monounsaturated fatty acids while

elovl2, *elovl5* and *elovl4* are PUFA elongases being selective for PUFAs (Leonard *et al.*, 2000; Tvrdik *et al.*, 2000; Moon *et al.*, 2001; Wang *et al.*, 2005; Cameron *et al.*, 2007; Tamura *et al.*, 2009). However, some elongases show a broad spectrum of substrate preference as well as end products. Such exceptional functions beyond the ranges of subfamilies suggest that the functional constraints of the subfamilies are not always effective. In particular, elongases seem to be more flexible about substrate specificities (Hashimoto *et al.*, 2008).

Of all elovl genes, *elovl1*, *elovl5* and *elovl6* are expressed ubiquitously whereas *elovl2*, *elovl3*, *elovl4* and *elovl7* display more distinctly tissue-specific levels of expression (Guillou *et al.*, 2010). However, the physiological consequences of such tissue-specific patterns of expression are yet to be elucidated. Although the key enzymes have now been identified, their functions and regulations are poorly understood. Of the different elovl genes, *elovl5* and *elovl2* are of particular interest because these genes participated in LC-PUFAs synthesis (Leonard *et al.*, 2000; Leonard *et al.*, 2002; Leonard *et al.*, 2004; Jakobsson *et al.*, 2006).

1.2.1 ELOVL family member 5 (*elovl5*)

The enzyme involving PUFAs elongation was first investigated in fungus (*M. alpina*) based on the review of the homologies shared by Jojoba β-ketoacyl-CoA synthase (KCS), *Arabidopsis* fatty acid elongation 1 and yeast elongase (*ELO1*, *ELO2*, *ELO3*) (Parker-Barnes *et al.*, 2000). Later, based on the similarity, although limited between yeast *ELO2* and human EST sequences, cDNA sequences human elongase 1 (*HELO1*) also known as *ELOVL5* that are involved in PUFAs elongation was identified (Leonard *et al.*, 2000). At the same time, Leonard and his research group found that a translated mouse EST sequence has 92.5% identity overlap with

HELO1 translated sequence which suggested a homologue of human *HELO1p* in mouse.

In 2004, elongase designated as *zfELO* was identified and isolated from zebrafish based on the search with *gLELO*, which encodes protein involved in elongation of PUFAs in fungus. The amino acid sequences of the open reading frame of *zfELO* shared high similarity with elongases of *Caenorhabditis elegans* and human (Agaba *et al.*, 2004). *zfELO* possessed the ability to convert C18, C20 and C22 PUFAs as well as the saturated and monounsaturated fatty acids which are similar to human *ELOVL5* (Leonard *et al.*, 2000) and rat *rELO1*(Inagaki *et al.*, 2002). Later, *zfELO* became more popularly known as *elovl5* because of its sequence similarity as well as the functional characteristic equivalent to human *ELOVL5* (Agaba *et al.*, 2005), Figure 1.4.

Numerous studies of *elovl5* have been carried out in various species. For example, tissue expression profiles of *elovl5* have been analyzed in rat (Wang *et al.*, 2005); atlantic salmon (Morais *et al.*, 2009); cobia (Zheng *et al.*, 2009); Asian sea bass (Mohd-Yusof *et al.*, 2010). Regulation of *elovl5* proved to be associated with PPARα, LXR, SREBP-1, ChREBP and MLX as well as to be influenced by dietary factors (Wang *et al.*, 2005; Wang *et al.*, 2006). Recently, *elovl5* knockout performed in mice were found to develop hepatic steatosis, with elevated levels of hepatic triacyglycerols via activation of SREBP-1c (Moon *et al.*, 2009). Role of *elovl5* in determination of *de novo* synthesis monounsaturated fatty acids have also been investigated through knockdown and overexpression of *elovl5* in mice by Green *et al.* (2010a).

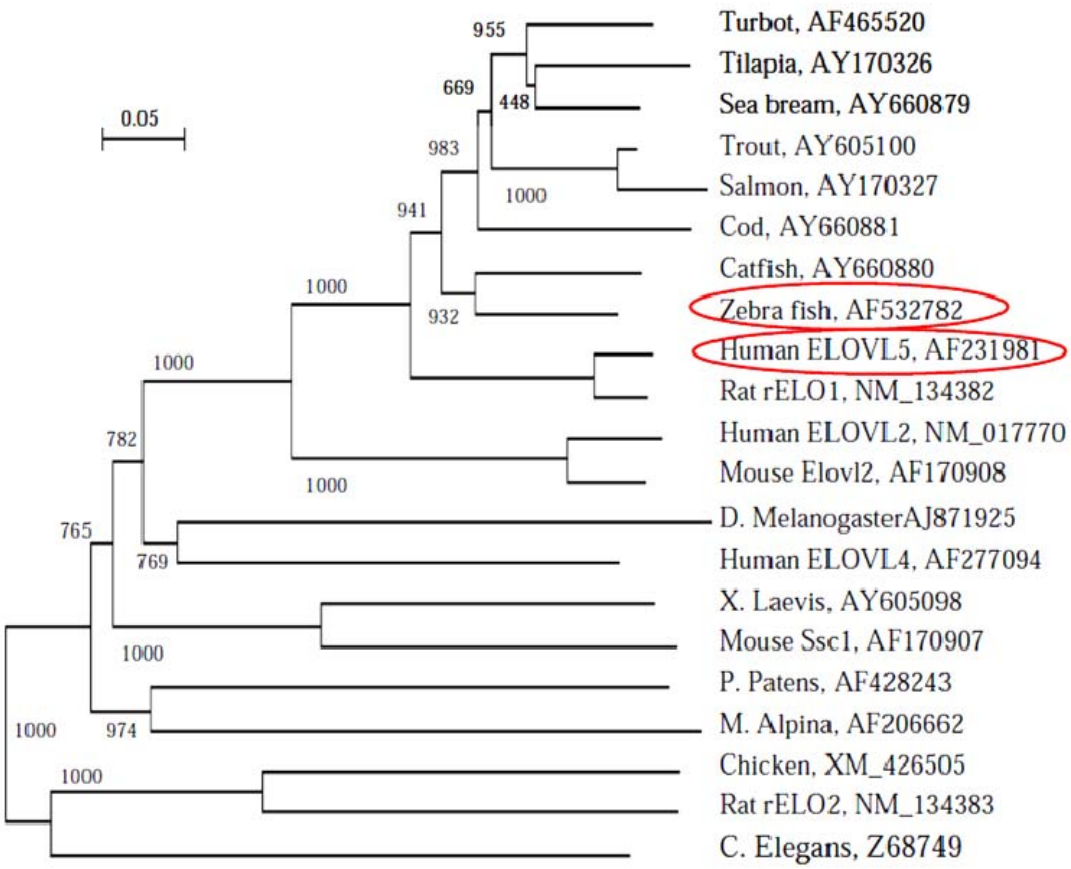

Figure 1.4 Phylogenetic relationships of elongases among various species. Amino acid sequences of elongases from various fish species (catfish, tilapia, turbot, seabream and cod) compare with elongases from other fish species (zebrafish and salmon), mammals (human, mouse and rat), avian (chicken), amphibian (*Xenopus laevis*), fungus (*Mortierella alpina*), nematode (*Caenorhabditis elegans*) and moss (*Physcomitrella patens*). Zebrafish elongase is closely related to human *ELOVL5*. Phylogenetic tree adapted from Agaba *et al.* (2005).

1.2.2 ELOVL family member 2 (*elovl2*)

Elovl2 was initially identified as *Ssc2* (sequence similarity to cig30-2) because of its sequence similarity to *Elovl3* (Cig30) (Tvrdik *et al.*, 2000). Studies in yeast showed that this enzyme may be involved in PUFAs elongation as it produced C24:4n-6 and C24:5n-3 (Moon *et al.*, 2001). *Elovl2* was first isolated from mammalian (human and mouse) using the *Elovl5* as query in search of enzymes

participating in PUFAs elongation (Leonard *et al.*, 2002). Expression of *Elovl2* was highest in the testis and liver, but the corresponding mRNA can also be detected at significant levels in the kidney, brain, lung and white adipose tissue (Tvrdik *et al.*, 2000; Wang *et al.*, 2005).

There is no *Elovl2*-like gene has been reported in nonmammalian vertebrates until 2009 when a second PUFA elovl gene was revealed in Atlantic salmon and zebrafish during searches in the GenBank, Atlantic salmon EST database and zebrafish genome. This gene was clearly found to be related to the mammalian *Elovl2* (Morais *et al.*, 2009). Later, Monroig and his research team isolated this gene from zebrafish designated as *elovl2* (Monroig *et al.*, 2009). Zebrafish *elovl2* was found to be closely related to salmon *elovl2* but was located at the distinct branch from zebrafish *elovl5* in the phylogenetic tree (refer Figure 1.5). Tissue distribution of *elovl2* in salmon was most abundant in intestine and liver, followed by brain (Morais *et al.*, 2009) while in zebrafish, the highest level of *elovl2* transcript was detected in liver, followed by intestine and brain (Monroig *et al.*, 2009). However, its spatio-temporal expression in zebrafish were fully resolved in this study.

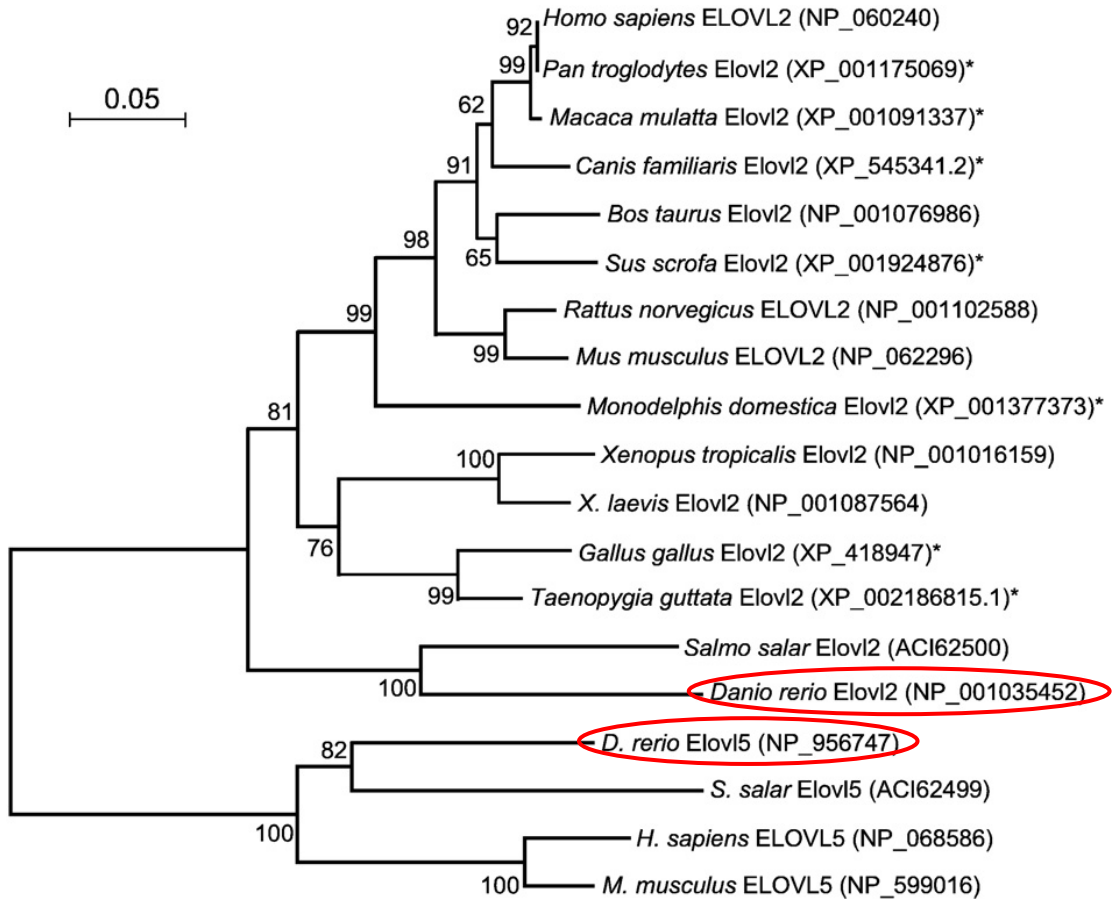

Figure 1.5 Phylogenetic tree comparing the putative zebrafish *elovl2*, *Elovl2* orthologs and *Elvol5* proteins. Zebrafish *elovl2* and *elovl5* were grouped into distinct clade. Figure modified from Monroig *et al.*(2009).

Elovl2 homologs to *Elovl5*, also possess ability to elongate C18 fatty acids, however, it shows highest activity towards C20 and C22. Unlike *Elovl5*, *Elovl2* is unable to elongate saturated and monounsaturated fatty acids. In addition, *Elovl2* was recently found to be involved in very long chain fatty acids (VLCFA) synthesis by controlling the level of C28:5n-6 and C30:5n-6 fatty acids in testis as a prerequisite for male fertility and sperm maturation in mice (Zadravec *et al.*, 2010). However, compensatory mechanism is suggested due to the redundant function between these two enzymes. In the *Elovl5* knockout mice, elongation activity of the *Elovl2* substrates C20:4n-6 and C20:5-3 was elevated whereby *Elovl2* try to counteract the reduction of C22:6n-3 in these mice (Moon *et al.*, 2009) indicating the compensation

of each other. In contrast to the case of *Elovl5*, there is no regulatory connection between *Elovl2* and SREBP-1c in rat hepatocytes (Wang *et al.*, 2006; Guillou *et al.*, 2010) even though it is known that PUFAs regulated SREBP-1c. Additionally, contradictory to *Elovl5*, expression of *Elovl2* in the rat liver is not influenced by fasting or refeeding or by dietary supplementation with fish or olive oils (Wang *et al.*, 2005).

1.3 Zebrafish (*Danio rerio*)

Zebrafish, scientifically named *Danio rerio*, is a tropical freshwater fish belonging to family of minnow or carp. Scientific classification of zebrafish is shown as below:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Danio*

Species: *D. rerio*

Zebrafish is named for the uniform, pigmented, horizontal black stripes on the side of the body, all of which extend to the end of the caudal fin. They are native to the streams of South-eastern Himalayan region (Talwar and Jhingran, 1991) and inhabit streams, canals, ditches, ponds, and slow-moving to stagnant water bodies, including rice fields (Spence *et al.*, 2008). They are small of about 3cm in length and commonly kept as aquarium fish. The males are slender and torpedo-shaped, with black longitudinal stripes with gold stripes in between. Males have gold colouration on the belly and fins. The females are fat and have larger, whitish belly and the

stripes in between black stripes are silver (Wixon, 2000). Life span of zebrafish is around 2 to 3 years, however, documented life span for laboratory zebrafish can exceed five years (Gerhard *et al.*, 2002).

Zebrafish is a common research model in fish studies of developmental biology (Driever *et al.*, 1994; Ingham, 1997), neurobiology (Appel, 2000), behavioral genetics (Gerlai, 2003; Sison *et al.*, 2006; Norton and Bally-Cuif, 2010) and drug screening (Parng *et al.*, 2002; Zon and Peterson, 2005; Sukardi *et al.*, 2011). Zebrafish emerge as complementary to other model organisms because it combines a number of key embryological and experimental advantages. According to Brand *et al.* (2002), zebrafish are easy to maintain and breed which is less demanding compared with keeping stock of mammals. Their constant supply of large numbers of offspring from defined pairs, renders the zebrafish ideally suited to large-scale genetic approaches aimed at identifying novel genes and uncovering their functions in a vertebrate (Pelegri, 2002). The establishment of zebrafish genome generated the genetic maps that facilitated novel gene identification. In addition, extensive homology of the zebrafish genome with other vertebrates including human, validated that the studies in zebrafish were relevant to vertebrate (Barbazuk *et al.*, 2000). Another advantage of studying zebrafish is that, it maintains the diploid state whereas other fish can be triploid or tetraploid, being an obstacle in genetic analysis (Wixon, 2000).

These advantages combined with recently established protocols and tools for genetic manipulation and analysis make the zebrafish uniquely suited to uncovering the genetic regulatory networks underlying the development and function of a vertebrate organism. As it is genetically more tractable, the aquaculture industry will likely be able to benefit significantly from insights gained from studying

zebrafish (Dahm and Geisler, 2006). Lately, zebrafish has been reviewed as a model organism for nutritional genomics studies to be applied on aquacultured fishes (Ulloa *et al.*, 2011) due to the most developed genomic program discovered in zebrafish compared to other aquacultured fish.

1.3.1 Zebrafish embryo

Zebrafish egg fertilization happens externally, allowing easy access to embryos for observation and manipulation. Owing to the transparency of zebrafish embryos, their internal organs can be easily studied under dissecting microscope. Moreover, the transparency of zebrafish embryo allows the easy visualization of internal processes as well as facilitates tracking the expression of fluorescently tagged transgene, monitoring reporter gene activity and laser manipulations (Gilmour *et al.*, 2002). Zebrafish embryos develop rapidly within 2-4 days (Wixon, 2000) shortening the time for investigation of zebrafish embryo development. Kimmel *et al*. (1995) defined zebrafish embryonic development into seven broad periods: the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching based on the morphological features. Studies of genetics and physiology during zebrafish embryogenesis was facilitated by knowledge of the morphogenesis and significant events that happen in each stage. In addition, studies on zebrafish embryos are applicable to aquacultured fish as well as to some extent in mammals in developmental biology.

In fish, studies have demonstrated that supply of LC-PUFAs to embryos is greatly influenced by the diet of brood stock (Rodríguez *et al.*, 1998; Almansa *et al.*, 1999; Mazorra *et al.*, 2003) and selective accumulation of certain lipid classes in the embryos has been proven (Tocher, 2003). In addition, various aspects of lipid metabolism in zebrafish has been recently revealed (Tocher, 2003; Ho *et al.*, 2004;

Schlegel and Stainier, 2006) rendering the research on lipid metabolism using zebrafish embryos remarkably relevant.

In addition, the development of morpholino-based antisense gene inhibition enabling specific gene knockdown opens the door to true functional genomics in zebrafish (Ekker, 2000; Ekker and Larson, 2001). Morpholino knockdown generates morphants with specific morphology which resembles the effect of loss of the particular gene. The optically clear zebrafish embryos provide *in vivo* image-based investigation of the effects of gene knockdown.

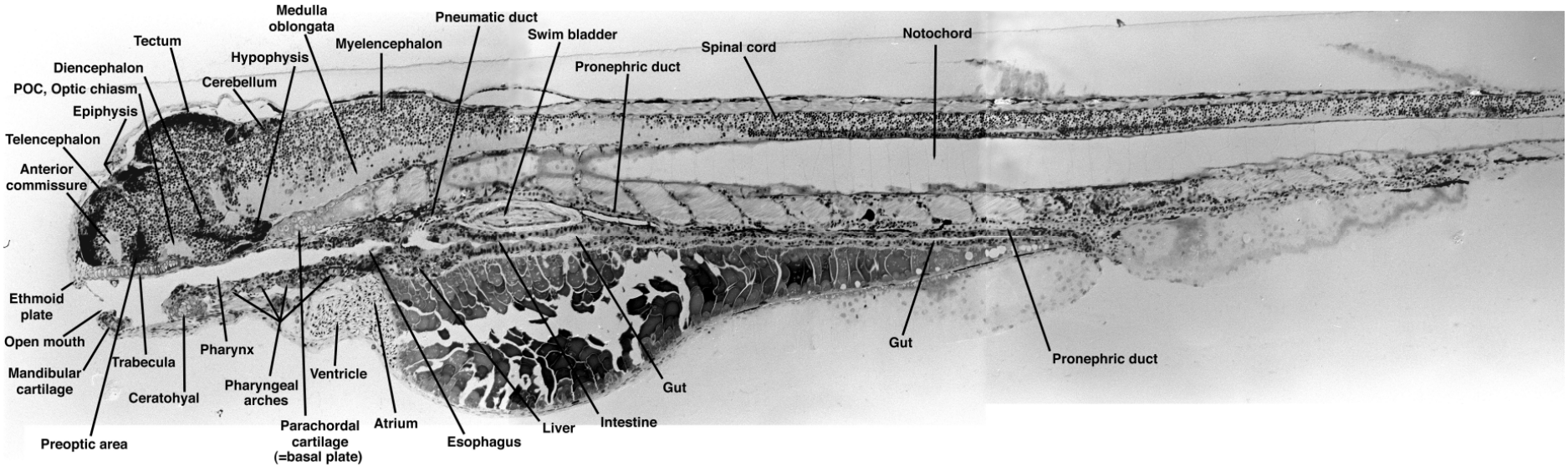

Figure 1.6 Anatomy of zebrafish embryo. Well characterized anatomy of zebrafish embryo assists identification of organs in gene expression study. Figure adapted from ZFIN (1996).

1.4 Objectives of study

Due to the importance of LC-PUFAs in various physiological events and the incapability of higher vertebrates like human to synthesize LC-PUFAs *de novo* in the body, it is necessary to investigate LC-PUFAs synthesis from their precursors in a broader spectrum. With the identification of key enzymes and establishment of LC-PUFA biosynthetic pathways in other species, a complete biosynthetic pathway of LC-PUFAs has been accomplished in zebrafish (Figure 1.7).

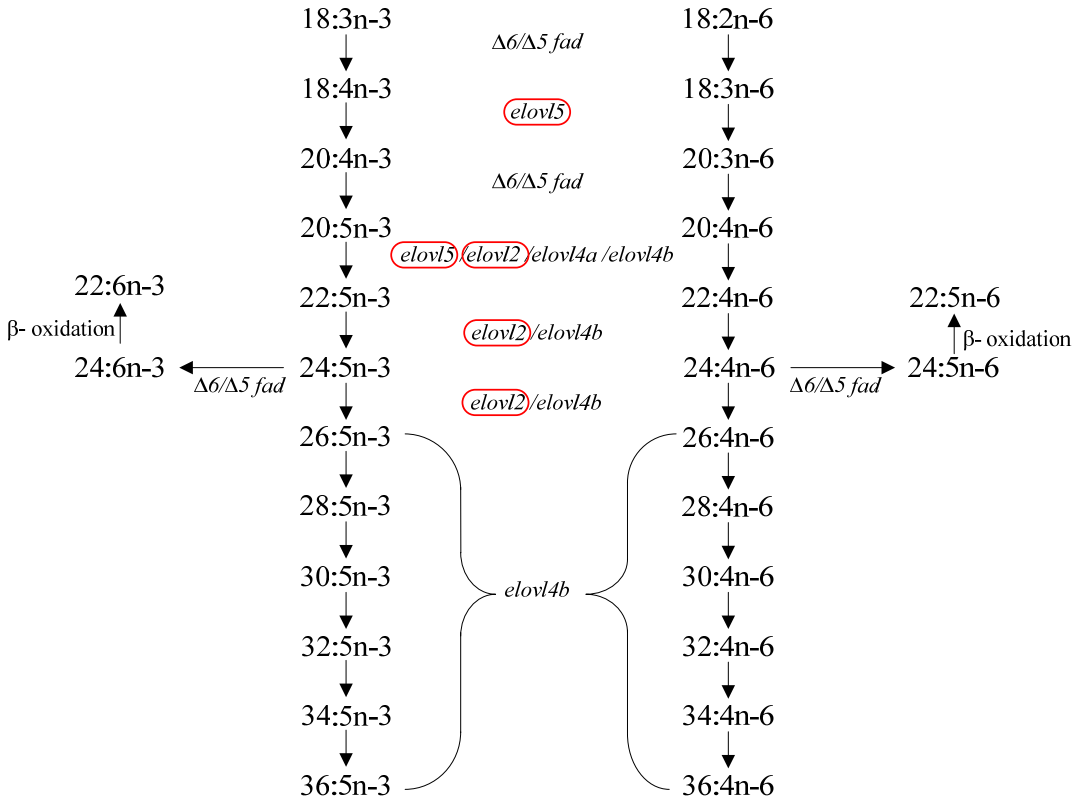

Figure 1.7 Long-chained polyunsaturated fatty acid biosynthetic pathway from n-3 and n-6 precursors in zebrafish. Enzymatic activities shown in the scheme are predicted from heterologous expression in *S. cerevisiae* of the dual Δ6/Δ5 fatty acyl desaturase (*Δ6/Δ5fad*) (Hastings *et al.*, 2001), *elovl5* (Agaba *et al.*, 2004) and *elovl2* (Monroig *et al.*, 2009) elongases.

As mentioned in sections 1.2.1 and 1.2.2, various studies had been conducted on *elovl5* and *elovl2* in different species. In zebrafish, investigations of these genes mainly in terms of their general expression profile and fatty acid contents

have been published (Monroig *et al.*, 2009). However, the expression of these two genes throughout the embryonic stages in temporal and spatial manner is still unknown. The expression profile of these genes can guide us to further investigate the function of these genes through identification of the organs and time of expression. With advanced tools that established in gene knockdown in zebrafish, we are able to inactivate the desired genes in order to define the function of these genes.

Even though *elovl5* knockout had been performed in mice (Moon *et al.*, 2009), but there is limitation using mouse as a research model. For instance, live imaging of the changes in the internal organ is not possible due to the opacity of mouse embryos. More importantly, this research is novel in zebrafish and permit the understanding of significant changes of internal organs across the different point of embryonic stages due to the knockdown. It is hoped that this pilot study will facilitate functional studies in higher vertebrate by determining the main organs involved. In addition, alteration of fatty acids content corresponding to the morphological changes would be useful to understand the necessity of the respective fatty acids in development and physiology of that particular organ.

Hence, this study utilizes the advantages of zebrafish embryos with the aid of technologies and tools to reveal the characters and functions of two important elongases in LC-PUFAs biosynthesis. The major objectives of the present study are:

I. To define spatio-temporal expression pattern of *elovl5* and *elovl2* throughout zebrafish embryogenesis.

II. To elucidate morphological and physiological consequences of knockdown of *elovl5* and *elovl2* each to the embryonic development.

III. To investigate the relationships of fatty acid profile changes to the effects of *elovl5* and *elovl2* knockdown.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The main material in this study is zebrafish embryos. The procedures to maintain zebrafish brood stock and collection of embryos are described as follow.

2.1.1 Fish maintenance

The wild type zebrafish were selected from population maintained in our lab since 2003 whereas the *ff1b* GFP transgenic line, *Tg(ff1bEx2:GFP)* was obtained from Prof. Chan Woon Khiong (National University of Singapore). Wild type zebrafish were used in all experiments in this study and *ff1b* GFP transgenic line was subjected to knockdown to verify the knockdown effect to the interrenal. The female and male zebrafish were kept in separate tanks in the Zebrafish Aquatic System (Aquaneering) in 14-hour photoperiod and 10-hour dark at the Aquaculture Research Complex. Fishes were fed until satiation twice daily with combination of brine shrimp and frozen bloodworms. Adult zebrafish aged 4 months were ready for mating for embryo sampling.

2.1.2 Embryo collection

Adult zebrafish were mated every 3 days in spawning tanks according to Westerfield (1995). Preparations for breeding were done in the afternoon where a pair of male and female fish was kept separately in the spawning tanks by a barrier. The barrier will be removed the next morning to allow the fishes to mate. The embryos were then cleaned and raised at 28.5ºC. Embryos were staged and collected under microscope at desired stages according to the hour post fertilization (hpf).

(Kimmel *et al.*, 1995). For whole-mount *in situ* hybridization, embryos were raised in distilled water supplemented with 0.003% N-phenylthiourea to prevent melanisation (Schulte-Merker, 2002).

2.2 Chemicals

Chemicals used in this study together with their respective suppliers are listed in Table 2.1.

Table 2.1 List of chemicals used in this study.

Chemicals	Suppliers
Agarose	Vivantis
DNA ladder (100bp)	Vivantis
iScript™ MMLV Reverse Transcriptase	Bio-Rad
pGEM®-T Easy Vector System	Promega
RQ1 RNase- Free DNase	Promega
SYBR® Green RT-PCR Reaction Mix	Bio-Rad
TRI Reagent®	Molecular Research Centre
QIAprep Spin Miniprep	Qiagen
NcoI restriction enzyme	NEB
NdeI restriction enzyme	NEB
NotI restriction enzyme	Fermentas
EcoRI restriction enzyme	Fermentas
SP6 polymerase	Roche
T7 polymerase	Roche
RNasin	Promega
DIG RNA labeling mix	Roche
Fluorescein RNA labeling mix	Roche
Anti-DIG antibody	Roche
Anti-fluorescein antibody	Roche
Fast Red	Roche
Paraformaldehyde	-
Blocking reagent	Roche

Table 2.1 Continued

Chemicals	Suppliers
Formamide	Fluka
Heparin	Sigma
Tween 20	Sigma
SSC	Sigma
Maleic acid	Sigma
Magnesium chloride	Amresco
Sodium chloride	R&M Chemicals
Tris	Bio-Rad
Ethyl 3-aminobenzoate	Sigma- Aldrich
Alexa Fluor-568 dextran	Molecular Probe
TEMED	Bio-Rad
SDS	Vivantis
Glycine	Vivantis
Methanol	Merck
Ammonium persulphate	USB Corporation
D-mannitol	Fluka
Elovl5 antibody (rabbit polyclonal)	Abcam
Elovl2 antibody (rabbit polyclonal)	Santa Cruz Biotechnology
Rabbit IgG-H&L (HRP) (goat polyclonal)	Abcam
PageRuler™ Prestained Protein Ladder	Fermentas

2.3 Lipid extraction and fatty acid analysis

In order to learn about how changes of elongase gene expression affect the fatty acid compositions during embryogenesis, fatty acid analysis was carried out on wild type embryos of 12, 24, 48, 72 and 120hpf. For fatty acid analysis of *elovl5* and *elovl2* morphants, 24, 48, 84 and 120hpf were collected. Total lipid was extracted by homogenization in solvent, chloroform: methanol (2:1, v/v) as described by Bligh and Dyer (1959). Lipid extract was methylated and transesterified with boron trifluoride in methanol. Fatty acid methyl esters (FAME) were then separated and quantified by gas chromatography (GC-2010, Shimadzu) equipped with flame ionization detector and a 30m × 0.22mm 70% Cyanopropyl Polysilphenylene-siloxane column (BPX70, SGE). Nitrogen was used as a carrier gas and temperature programming was from 100 to 210ºC at 2 ºC increase per minute, and then held at 210 ºC for 10 minutes. The injector and detector temperatures were set at 250 and 260 ºC, respectively. Individual methyl esters were identified by comparison to known standards and by reference to Ackman (1980). Compositions of substrate and product fatty acids were calculated as percentage of total fatty acids.

CHAPTER 3

SPATIO-TEMPORAL EXPRESSION OF *ELOVL5* AND *ELOVL2* DURING ZEBRAFISH DEVELOPMENT

3.1 Introduction

Long-chained polyunsaturated fatty acids (LC-PUFAs), particularly n-3 and n-6 PUFAs are recognized as essential nutrients. The representative fatty acid of LC-PUFAs are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) which have been intensively investigated in many studies. Studies revealed such LC-PUFAs present beneficial effects on health, for example in reducing cardiovascular diseases (Calder, 2004; Breslow, 2006); inflammatory and autoimmune diseases (Simopoulos, 2002; Calder, 2006) and retinal diseases (SanGiovanni and Chew, 2005; Schnebelen *et al.*, 2009). In addition, LC-PUFAs are crucial in reproduction as well as during ontogenesis (Wathes *et al.*, 2007; Wakefield *et al.*, 2008; Tocher, 2010).

Though LC-PUFAs possess diversity of biological functions, knowledge on the mechanism and regulation of their synthesis, assimilation and metabolism in lipid-rich tissues during development require further elucidation. A key approach to unravel the requirement of LC-PUFAs during development will be to understand the activities and regulation of the relevant enzymes in LC-PUFAs biosynthesis. Biosynthesis of LC-PUFAs from their respective precursors involve a number of enzymes through a series of conversion activities. Two types of enzymes participate in the conversion, namely desaturase and elongase, involved in insertion of double bonds and at specific carbon atom and addition of two carbon atoms in fatty acid chain respectively. In the past decade, a great extent of studies had been done on