Elovl4a participates in LC-PUFA biosynthesis and is regulated by PPARαβ in golden pompano *Trachinotus ovatus* (Linnaeus 1758)

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The elongases of very long-chain fatty acids (Elovls) are responsible for the rate-limiting elongation process in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis. The transcription factor, PPARαβ, regulates lipid metabolism in mammals; however, the detailed mechanism whereby PPARαβ regulates Elovls remains largely unknown in fish. In the present study, we report the full length cDNA sequence of *Trachinotus ovatus* Elovl4a (*ToElovl4a*), which encodes a 320 amino acid polypeptide that possesses five putative membrane-spanning domains, a conserved HXXHH histidine motif and an ER retrieval signal. Phylogenetic analysis revealed that the deduced protein of *ToElovl4a* is highly conserved with the *Oreochromis niloticus* corresponding homologue. Moreover, functional characterization by heterologous expression in yeast indicated that *ToElovl4a* can elongate C18 up to C20 polyunsaturated fatty acids. A nutritional study showed that the protein expressions of *ToElovl4a* in the brain and liver were not significantly affected among the different treatments. The region from PGL3-basic-Elovl4a-5 (−148 bp to +258 bp) is defined as the core promoter via a progressive deletion mutation of *ToElovl4a*. The results from promoter activity assays suggest that *ToElovl4a* transcription is positively regulated by PPARαβ. Mutation analyses indicated that the M2 binding site of PPARαβ is functionally important for protein binding, and transcriptional activity of the *ToElovl4a* promoter significantly decreased after targeted mutation. Furthermore, PPARαβ RNA interference reduced *ToPPARαβ* and *ToElovl4a* expression at the protein levels in a time-dependent manner. In summary, PPARαβ may promote the biosynthesis of LC-PUFA by regulating *ToElovl4a* expression in fish.

Long-chain polyunsaturated fatty acids (LC-PUFA) are involved in numerous biological processes and are major components of complex lipid molecules1. In vertebrates, two LC-PUFA biosynthetic pathways are defined: the “Δ6 pathway” (Δ6 desaturation-elongation-Δ5 desaturation) and the “Δ8 pathway” (elongation-Δ8 desaturation-Δ5 desaturation); these are initiated from α-linolenic (18:3n-3) and linoleic (18:2n-6) acids, respectively1–5. Two sets of enzymes, the elongases of very long-chain fatty acids (Elovls) and fatty acyl desaturases (Fads), are involved in these pathways6. The Elovls protein family include seven isozymes (Elovl1-7) in vertebrates7. In *vitro* FA elongation assays, knockdown and knockout (KO) of Elovl1-7 genes revealed that Elovl1-7 exhibits substrate specificity; each isozyme prefers acyl-CoAs with specific chain-lengths and/or a degree of saturation8. The over-expression of fish elongases has also elevated the endogenous production of LC-PUFA in fish species, such as transgenic *Danio rerio* and *Miichthys miui*9,10. The Elovl4 enzyme has been widely studied in teleosts, especially in marine...
date the transcriptional regulation of PPAR α

ToElovl4a the identification of α function and showed that PPAR α promoter. Finally, the suppression of expression (RNAi) of PPAR α promoter activity assays via the mutation of potential PPAR α ToElovl4a transcriptional characterization of the gene was performed using heterologous expression in yeast. Second, α focused on clarifying the importance of PPAR β in regulating b during LC-PUFA biosynthesis, the present study showed three conserved domains, which contained five putative membrane-spanning domains with a conserved HXXHH histidine motif and an ER retrieval signal (Fig. 1). The phylogenetic tree analysis indicated that b performs a vital function in the regulation of O. niloticus, D. rerio, H. sapiens and G. gallus Elovl4. Five (I–V) putative membrane-spanning domains are indicated by yellow colour. The conserved HXXHH histidine motif and ER retrieval signal are indicated by yellow and blue boxes, respectively. Dashes represent gaps created to maximize the degree of identity among all compared sequences. The accession numbers of the sequences used are from Supplementary Table 3.

Compared sequences. The accession numbers of the sequences used are from Supplementary Table 3. Yeast heterologous expression systems revealed that Elovl4 is mainly involved in the elongation of C20–22 LC-PUFA, producing polyenes of up to 36 carbons in the biosynthetic pathway of LC-PUFA1,12,16. Peroxisome proliferator-activated receptor alpha (PPARα) is a member of the steroid receptor superfamily of ligand-activated nuclear transcription factors and is known to regulate lipid and glucose metabolism17,18. Furthermore, PPARα stimulates the expression of target genes via direct binding to PPAR response elements (PPREs) in the promoter region of target genes19,20. It has been shown that PPARα upregulates Fads2 promoter activity in fish and avians21,22. Both PPARα1 and PPARα2 were found to activate the promoter activity of Fads2 in Lateolabrax japonicas; however, no such regulatory activity was detected for Linmaichthys crocea23.

The golden pompano Trachinotus ovatus (Linnaeus 1758), Carangidae, and Perciformes are found in the Asia-Pacific region and are considered important aquaculture fish in China because of their economic value23,24. Furthermore, the T. ovatus muscle has been found to be rich in PUFA (such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) after feeding without PUFA (EPA and DHA)25, showing that it has the ability to endogenously compound PUFA. Consequently, T. ovatus provides an exceptional model for the investigation of regulatory mechanisms in LC-PUFA biosynthesis in teleosts. To investigate the underlying function of T. ovatus Elovl4a (ToElovl4a) and the regulation of Elovl4a by PPARα during LC-PUFA biosynthesis, the present study focused on clarifying the importance of PPARα in regulating ToElovl4a transcriptional activity. First, a functional characterization of the ToElovl4a gene was performed using heterologous expression in yeast, the suppression of expression (RNAi) of PPARα was used to elucidate the transcriptional regulation of PPARα with respect to ToElovl4a. These approaches have contributed to the identification of ToElovl4a function and showed that PPARα performs a vital function in the regulation of Elovl4a expression.

Results

Molecular cloning and phylogenetics of T. ovatus Elovl4a. The T. ovatus putative elongase full length cDNA was 1,606 bp and included an ORF of 963 bp. This nucleotide sequence translated to a peptide sequence of 320 amino acids (Accession no. MG674424) (Supplementary Fig. 1). A BLAST analysis revealed that the ToElovl4a protein sequence shared high sequence identity with Elovl4a sequences from other teleosts, including tilapia (Oreochromis niloticus, 96%, Ensembl No. ENSONIP00000009904.1) and zebrafish (Danio rerio, 81%, Ensembl No. ENSDARG0000006773), shared low sequence identity with chicken (Gallus gallus, 68%, Ensembl No. ENSGALG0000015876) and humans (Homo sapiens, 64%, Ensembl No. ENSG00000118402).

Interestingly, comparisons of the amino acid sequences for the Elovl4a protein from the above four species showed three conserved domains, which contain conserved putative membrane-spanning domains with a conserved HXXHH histidine motif and an ER retrieval signal (Fig. 1). The phylogenetic tree analysis indicated that ToElovl4a clustered with several other Elovl4a sequences from other osteichthyes, and more distantly, with avian (G. gallus) and mammalian (H. sapiens) Elovl4a (Fig. 2). ToElovl4a was grouped together with perciformes, such as O. niloticus.

Heterologous expression of the elongase ORF in Saccharomyces cerevisiae. The function of ToElovl4a was characterized by determining the FA profiles in S. cerevisiae, was transformed with pYES2-Elovl4a

| Species | Accession No. | Sequence Identity | Ensembl No. |
|---------|---------------|------------------|-------------|
| T. ovatus | ENSDARG00000006773 | 96% | 1,606 bp |
| O. niloticus | ENSONIP00000009904.1 | 68% | 1,586 bp |
| D. rerio | ENSG00000118402 | 64% | 1,586 bp |
| G. gallus | ENSGALG0000015876 | 64% | 1,586 bp |
and was grown in the presence of potential FA substrates, including C18 (18:2n-6, 18:3n-3, 18:3n-6 and 18:4n-3), C20 (20:4n-6 and 20:5n-3) and C22 (22:5n-3, 22:4n-6 and 22:6n-3) substrates. In yeast transformed with pYES2-Elolv4a and grown in the presence of 18:3n-6 (Fig. 3A), however, an additional FA peak was identified as 20:3n-6 (Fig. 3B) based on the gas chromatography (GC) retention times. Therefore, from this data, it was concluded that the ToElov4a can efficiently elongate C18 up to C20. The conversion rates of 18:3n-6 to 20:3n-6 were calculated to be approximately 1.05% (Table 1). Moreover, upon comparison with the gas mass spectrometry database, our results indicated that no other FA mass spectrometry structures were detected except for the 20:3n-6 structure.

Figure 2. Phylogenetic relationship of T. ovatus Elolv4a/b amino acid sequences with their counterparts from other species. The main topology was produced by MEGA 6 software with the maximum likelihood (ML) method with 1000 bootstrap replicates. The accession numbers of the sequences used are from Supplementary Table 3.

Figure 3. Functional characterization of the putative Elolv4a in transgenic yeast. (A and B) represent adding polyunsaturated fatty acid (FA) substrate of C18:3n-6. FAs were extracted from yeast transformed with the pYES2 vector, including the ORF of the putative Elolv4a cDNA as an insert. Peaks 1–4 represent the main endogenous FAs of T. ovatus, namely, C16:0, C16:1 isomers, C18:0 and C18:1n-9, respectively. Based in the retention times, additional peaks were identified as 20:3n-6 (B). Vertical axis, FID response; horizontal axis, retention time.

Tissue distribution of ToElov4a. Tissue distributions of ToElov4a were delineated by qRT-PCR. The highest ToElov4a mRNA levels were detected in the brain, followed by the stomach and intestine, whereas...
relatively low ToElovl4a expression levels were observed in the liver and spleen (Fig. 4). Notably, the expression of ToElovl4a in the brain was much higher than in other tissues ($P < 0.05$).

**Nutritional regulation of ToElovl4a.** The protein expression of ToElovl4a in the liver and brain fed with different levels of LNA or LA (18:3n-3 or 18:2n-6) through the diet was determined by a western blot. The GAPDH was used as an internal control for normalization. The express pattern of ToElovl4a protein levels in the liver and brain were uncorrelated with the fatty acid compositions (Fig. 5) (also Supplementary Fig. S1).

**Promoter analysis of PPARαβ regulation.** The cloned candidate ToElovl4a promoter (1,057 bp) was an upstream non-transcribed sequence. To determine the binding region of PPARαβ in the ToElovl4a promoter, a full length candidate promoter and several truncated mutants were constructed with a promoterless luciferase reporter vector, pGL3-basic. The promoter construct, Elovl4a-p5 (−148 bp to +258 bp), exhibited the highest promoter activity with PPARαβ, suggesting that this region of the Elovl4a-p5 promoter sequence contained the PPARαβ binding site (Fig. 6A).

To further confirm the interaction of ToPPARαβ with ToElovl4a, the influence of ToPPARαβ overexpression on ToElovl4a transcription was determined. PPARαβ overexpression increased the promoter activity of ToElovl4a-5 at all tested time points in heterologous HEK 293 T cells, and the maximum difference occurred at 12 h posttransfection, which was 5.6-fold higher in the PPARαβ-overexpressing cells than that in the controls (Fig. 6B). These results indicated that constitutively expressed PPARαβ positively regulated ToElovl4a expression in HEK 293 T cells.

To identify the PPARαβ binding sites in the Elovl4a promoter, the predicted binding sites were mutated (Fig. 7, Table 2). The effects on promoter activity were investigated in 293 T cells that were transfected with each mutant and PPARαβ. The results revealed that mutation of the M2 binding site (+209 bp to +223 bp) caused significant reduction in promoter activity (Fig. 7), showing that M2 was the PPARαβ binding site in the Elovl4a promoter. Notably, three other predicted binding sites did not induce luciferase activity with PPARαβ, suggesting that these three sites were not required for triggering ToElovl4a expression with PPARαβ.

**Transcriptional regulation of ToElovl4a by PPARαβ.** Protein levels of ToPPARαβ were considerably decreased in a time-dependent manner by the RNAi of PPARαβ, suggesting effective knockdown of ToPPARαβ expression in T. ovatus caudal fin cells (TOCF) (Fig. 8A) (also Supplementary Fig. S2A). When ToPPARαβ expression was reduced, the protein levels of ToElovl4a were considerably depleted compared with the control.

### Table 1. Conversion rates of pYES2-Elovl4a transformed yeast grown in presence of 18:3n-6 substrate.

| FA substrate | Product | Conversion (%) | Activity |
|--------------|---------|----------------|----------|
| 18:2n-6      | —       | 0              | —        |
| 18:3n-3      | —       | 0              | —        |
| 18:3n-6      | 20:3n-6 | 1.05%          | C18 → C20 |
| 18:4n-3      | —       | 0              | —        |
| 20:4n-6      | —       | 0              | —        |
| 20:5n-3      | —       | 0              | —        |
| 22:5n-3      | —       | 0              | —        |
| 22:4n-6      | —       | 0              | —        |
| 22:6n-3      | —       | 0              | —        |

Figure 4. Gene transcriptions of Elovl4a in various tissues of T. ovatus. The twelve tissues are brain (Br), stomach (St), intestine (In), male gonad (Mg) and female gonad (Fg), fin (Fi), blood (Bl), kidney (Ki), gill (Gi), white muscle (Wm), liver (Li), and spleen (Sp). Significant differences at $P < 0.05$ are labelled with different letters, and mean ± SEM of each mRNA quantity is shown for each tissues tested.
group at corresponding time points (Fig. 8B) (also Supplementary Fig. S2B). These results suggested an active regulatory role of ToPPAR\(\alpha\)b on ToElovl4a expression in the TOCF cells.

**Discussion**

The present study sought to gain insights into the mechanisms underlying the transcriptional regulation of LC-PUFA biosynthesis in *T. ovatus*. To achieve this, sequence and functional characterization, tissue expression patterns and transcriptional regulation of ToElovl4a were investigated. The ToElovl4a ORF encodes a protein that is 81%–96% identical to Elovl4 proteins from other teleosts. These isolated ToElovl4a proteins contain three classic structural motifs, including transmembrane domains, a conserved histidine box (HXXHH), and an ER retrieval signal (RXKXX) in the canonical C-terminal, indicating its specific role is in LC-PUFA biosynthesis\(^{26}\). These three conserved boxes were also found to be present in other species Elovl4 proteins\(^{11–15}\). The ToElovl4 sequence is positioned within the teleost Elovl4 clade together with *O. niloticus*, and the teleost Elovl4 clade is outgrouped by the tetrapod Elovl4 clade containing the Elovl4 sequences from avians and mammals.

Three members of the fatty acid elongases protein family, Elovl2, Elovl4 and Elovl5, have been described as crucial enzymes involved in the biosynthetic pathway of LC-PUFA in teleosts\(^{127}\). For marine fish, yeast heterologous expression systems indicated that Elovl5 can effectively elongate both C18 and C20 PUFA, whereas Elovl4 is mainly involved in the elongation of C20–22 LC-PUFA producing polyenes up to 36 carbons\(^{113}\). However, it
was also revealed that Elovl4 proteins are able to utilize all assayed C18–22 PUFA substrates. In this study, the functional characteristics of ToElovl4a via heterologous expression in *S. cerevisiae* showed that the *T. ovatus* putative elongase is Elovl4a, which can only elongate C18 (18:3n-6) substrates to C20 (20:3n-6) PUFA. In agreement with the functional data obtained for some marine fish, such as the 7.6% low activity in *Scatophagus argus* 13, the 4.6% in *Acanthopagrus schlegelii* 14, and the 6.1% in *Larimichthys crocea* 15, ToElovl4 also showed low activity (1.05%) towards PUFA substrates, which confirmed its role in the biosynthesis of VLC-PUFA. However, until now, unlike the present study, Elovl4a was also found to effectively convert C18-C22 PUFA to longer polyenoic products up to C36 in other carnivorous fish 1,11–15, suggesting that marine fish Elovl4 exhibited high elongation efficiency towards C18-C22 PUFA substrates, except ToElovl4a. It is inferred that ToElovl4a solely elongates omega-6 C18 fatty acids, and this has been hypothesized as an adaptive strategy to supplement for Elovl5 in *T. ovatus*. For *T. ovatus*, yeast heterologous expression systems showed that Elovl5 can effectively transform C18-C20 PUFA and ToFads6 that possess Δ4/Δ5/Δ8 Fad desaturation activity 27,28. In addition to the present study, thus far, the complete classical pathways of LC-PUFA biosynthesis have not been elucidated for *T. ovatus*.

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**Table 2.** Primers used for site-directed mutations of putative binding sites on ToElovl4a promoter.

| Putative binding sites | Nucleotide sequence | Mutated pattern |
|------------------------|--------------------|-----------------|
| M1                     | CCAGAGAAAGCCCA     | deletion        |
| M2                     | TCATTTCAGCTCCAC    | deletion        |
| M3                     | ACACATGGCGCTCTGCC  | deletion        |
| M4                     | CTGGCCGCTGGGACTGT  | deletion        |

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**Figure 7.** The nucleotide sequence and predicted binding sites for transcription factors in the core region of the ToElovl4a promoter (A). Effects of transcription factor mutations on ToElovl4a-5 promoter activity (B). Binding sites are shown with boxes. Mutations of promoter sequences are listed in Table 2. All values are presented as the means ± SD (n = 3). Asterisks indicate that the values are memorably different from the individual controls (✳p < 0.05 and ✲p < 0.01). Bars on the same group with different letters are statistically significant from one another.

**Figure 8.** ToPPARα upregulates ToElovl4a expression. Western blot analysis was used to detect the expression of ToPPARα (A) and ToElovl4a (B) after the transfection of either control RNA (Control) or siRNA (RNAi), respectively. Full-length blots are presented in Supplementary Fig. S3.
In the present study, the highest ToElovl4a mRNA expression was detected in the brain, showing that essential fatty acid metabolism occurs in the brain. However, relatively moderate ToElovl4a mRNA expression levels were detected in the stomach, intestine and gonad. Interestingly, these are the first tissues exposed to dietary lipids, and they are the main lipid metabolism tissues in the body. Moreover, the liver is the main site for LC-PUFA synthesis. These studies indicate that lower levels of hepatic Elovl4a transcripts in carnivorous marine fish, like T. ovatus, may correlate with their limited LC-PUFA biosynthetic abilities.

Previous studies have indicated that Fad enzymatic activity and gene expression vary with dietary LNA/LA (18:3n-3/18:2n-6) ratio. Upregulation of Δ6 Fads2 gene expression was detected in Siganus fuscescens, Maccullochella pedeli, Oncorhynchus mykiss and Scatophagus argus that were fed high dietary ratios of LNA/LA. Unlike desaturases, there is a lack of data on the influence of dietary LNA/LA ratio on elongase expression. Xie et al. showed that the expression of Elovl4 and Elovl5 is significantly affected by dietary fatty acid composition, and they showed the highest expression of mRNA in the liver and eye of fish fed a diet that contained LNA/LA in a ratio of 1.7:1 in Scatophagus argus. Unfortunately, in the present nutritional experiment, no pattern was found between the expression of ToElovl4a and fatty acid composition.

In general, mRNA levels of some genes in eukaryotic cells are dependent on transcription factors and RNA polymerases binding to specific sequences in gene promoters. Consequently, the integrity and activity of a promoter can affect the gene expression. Moreover, PPARs are ligand-activated transcription factors that are necessary for regulating gene expression in the PUFA biosynthesis pathway. Dual luciferase reporter assays were conducted to clarify regulatory mechanisms whereby PPARα is believed to modulate Elovl4a expression. Analysis of the truncated mutants indicated that ToElovl4a reporter activity was induced by the overexpression of PPARα. The core binding region in the ToElovl4a promoter is -148 bp to -258 bp (Fig. 6A). This was the first evidence showing that the transcription of Elovl4a may be upregulated by PPARα. In a previous study, PPARα interacted with the binding site of the ToElovl5 and ToFads6 promoter region to positively regulate ToElovl5 and ToFads6 transcription, respectively. Obviously, PPARα plays a key regulatory role in the LC-PUFA biosynthesis in T. ovatus. Furthermore, the deletion of the PPARα M2 binding site (+209 bp to +223 bp) results in significantly reduced promoter activity (Fig. 7). To further confirm whether PPARα is a transcription factor implicated in ToElovl4a function, the effects of PPARα knockdown on ToElovl4a protein expression were investigated by western blotting in TOCF cells. These data showed that PPARα upregulated ToElovl4a protein levels.

In summary, the functional studies presented here show that ToElovl4a may effectively extend 18:3n-6 substrates. Moreover, the proposed synthesis pathway of LC-PUFA was for T. ovatus. Furthermore, we demonstrated clear associations between PPARα and the ToElovl4a promoter and the positive regulatory functions of PPARα in ToElovl4a transcription. These results provide new insights into the regulation and function of Elovl4a in fish and further reveal the complexity of the associated regulatory mechanisms.

Materials and Methods

Ethics statement. All experiments in this study were approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. SCSFRI96-253) and were performed according to the regulations and guidelines established by this committee. To minimize suffering of the fish, all surgeries were implemented with 0.01% 2-phenoxyethanol (Sigma-Aldrich) anaesthesia.

Diets, fish, feeding trial and sampling. Eight isonitrogenous and iso-lipidic diets were formulated with 45% crude protein and 12% crude lipid with different lipid sources (Supplementary Table 1). Diet 1 contained fish oil (FO) as the control, and diets 2–8 contained different proportions of fish oil, krill oil, soybean oil and corn oil. The dietary formulations, proximate and fatty acid compositions are shown in Supplementary Table 1.

To determine the tissue expression profile of ToElovl4a, healthy fish tissue (n = 6) containing small intestine, liver, white muscle, brain, spleen, fin, gill, head kidney, stomach, blood, males and female gonads were sampled, flash frozen in liquid nitrogen, and stored at -80 °C until further use.

Gene cloning and bioinformatics of ToElovl4a. Total RNA (1 μg) was extracted from T. ovatus brain by TRIzol Reagent (Takara, Japan). The quality and quantity (concentration) of isolated RNA were determined using a NANO DROP 2000 spectrophotometer (Thermo Scientific). Subsequently, cDNA was synthesized using the PrimeScript™ RT reagent kit (Takara, Kyoto, Japan), according to the manufacturer’s instructions. A putative ToElovl4a number was derived from the annotation file of T. ovatus. Subsequently, a putative ToElovl4a sequence was obtained based on CDS data of T. ovatus. (https://doi.org/10.6084/m9.figshare.7570727.v1 (2019)). To determine the veracity of the putative Elovl4a sequence, gene-specific primers were designed (Supplementary Table 2). The PCR protocol used has been previously described. The amplified products were purified by a DNA purification kit (Tiangen, China), ligated into the pEASY-T1 vector (TransGen Biotech, China), and sequenced (Invitrogen, Guanzhoun, China). Validated plasmids were transformed into competent Trans1-T1 cells (TransGen Biotech, China). A Blast search on the putative Elovl4a ORF sequence further confirmed the accuracy and validity.

The deduced amino acid sequence of the cloned ToElovl4a open reading frame (ORF) was aligned with other Elovl4 orthologue ORFs (Supplementary Table 3). Multiple sequence alignments were conducted using ClustalX.

In summary, the functional studies presented here show that ToElovl4a may effectively extend 18:3n-6 substrates. Moreover, the proposed synthesis pathway of LC-PUFA was for T. ovatus. Furthermore, we demonstrated clear associations between PPARα and the ToElovl4a promoter and the positive regulatory functions of PPARα in ToElovl4a transcription. These results provide new insights into the regulation and function of Elovl4a in fish and further reveal the complexity of the associated regulatory mechanisms.
portion of the calibration curve with PCR efficiency for 20 s. Amplification efficiencies of the target and reference genes were observed from the slope of the log-linear standard methods. Once generated, the polyclonal antibody was pre-adsorbed using to generate a polyclonal antibody, purified rToElovl4a protein was injected into white New Zealand rabbits using an AKTAprime™ Plus system (GE Healthcare, USA). The programme parameters were 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 10 min. The total protein was extracted using ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich) and electrophoresed on 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) using the PierceG2 Fast Blotter (25 V for 10 min, Pierce, Rockford, IL, USA). Western blotting analyses was executed according to a previously described protocol. To confirm specificity of the rabbit anti-Elovl4a antibody, human embryonic kidney (HEK293T) cells were transfected with pcDNA3.1 and pcDNA3.1-Elovl4a for 48 h. After this period, cells were harvested by centrifugation at 160 g for 10 min at 4 °C. The total protein was extracted using ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich). Then, the total protein were electrophoresed on 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) using the PierceG2 Fast Blotter (25 V for 10 min, Pierce, Rockford, IL, USA). Western blotting analyses was executed according to a previously described protocol.

To observe the endogenous Elovl4a expression, T. oivatus caudal fin (TOCF) cells were cultured in six-well plates at a density of 2.5 × 10⁶ cells/well. After the TOCF cells were transfected with PPARαb siRNA, cells were harvested and lysed as described above. Then, the total protein was incubated with/without calf intestinal alkaline phosphatase (CIAP) (20 U) at 37 °C for 30 min, separated by 12% SDS-PAGE and transferred to PVDF membranes using the PierceG2 Fast Blotter (25 V for 10 min; Pierce, Rockford, IL, USA). Primary antibodies [anti-Elovl4a, murine anti-Flag (Sigma-Aldrich, St. Louis, MO, USA) and the loading control, the anti-glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH; Sigma-Aldrich), 1:1000] were incubated with the PVDF membrane in 1% (w/v) non-fat milk in Tris-buffered saline and Tween 20 (TBST) buffer (0.1% Tween 20) for 3 h. Horse radish peroxidase-(HRP)-conjugated goat anti-rabbit antibody (1:3000) was used as a secondary antibody (Sigma-Aldrich). The results were observed using an electrochemiluminescence (ECL) system.
was isolated at specific time points (0 h, 6 h, 12 h, and 24 h) as described above. PPAR and INhe Detection of promoter activities were at specific time points (0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h). The siRNA for α promoter segment were chosen to determine the regulatory relationship between ToPPAR α and the same restriction endonucleases (Takara, Japan) and ligated into a correspondingly restricted pCDNA3.1-Flag vector (Invitrogen, USA). Transfection reporter assays were described by Li et al. Relative luciferase activities (firefly and renilla luciferase activities) were measured by a VICTOR™ X2 Multi-label Plate Reader (PerkinElmer, Inc., Waltham, MA, USA).

TOCF cells were cultured in L15 media (Gibco, USA) supplemented with 10% FBS, 100 μg mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin at 28 °C. Before DNA transfection, cells were seeded in 24-well plates until they were 90–100% confluent. Then, small interfering RNA (siRNA) or plasmids were transfected using Lipofectamine RNAiMAX or Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions.

Expression analysis of ToElovl4α with ToPPARα. The ORF of T. ovatus PPARβ (ToPPARα) (GenBank accession number: MH321826) was amplified with primers incorporating restriction sites for Nhe I and Hind III at the 5' and 3' ends, respectively (Supplementary Table 2). The DNA fragment was digested with the same restriction endonucleases (Nhe I and Hind III; Takara, Japan) and ligated into a correspondingly restricted pCDNA3.1-Flag vector (Invitrogen, USA). Transcription factors ToPPARb and pGL3-basic-Elovl4a-5 of the promoter segment were chosen to determine the regulatory relationship between ToPPARb and ToElovl4α. Detection of promoter activity were at specific time points (0 h, 3 h, 6 h, 12h, 24h, 48h and 72h). The siRNA for PPARb (PPARb-si) and the negative control (si-NC) were purchased from Genecreate (Wuhan, China). The PPARb siRNA sequence is listed in Supplementary Table 2. After transfection with TOCF cells, the total protein was isolated at specific time points (0 h, 6 h, 12 h, and 24 h) as described above.

Statistical analysis. SPSS 19.0 software (IBM, USA) was used to conduct the statistical analyses. The data were analysed by the Duncan test using one-way ANOVA. All data from the relative expression represented at least three replications along with means ± standard error of the mean (SE). Differences were considered significant at the p < 0.05 level.

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K.C.Z., S.G.J. and D.C.Z. designed the research and wrote the paper. S.L. and K.C.Z. performed the research. H.Y.G. and N.Z. analyzed the data. B.S.L. and L.G. contributed reagents/materials/analysis tools.

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