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Divergent evolution of *cis*-acting peroxisome proliferator-activated receptor elements that differentially control the tandemly-duplicated fatty acid-binding protein genes, *fabp1b.1* and *fabp1b.2*, in zebrafish

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Draft

Abstract

Gene duplication is thought to facilitate increasing complexity in the evolution of life. The fate of most duplicated genes is nonfunctionalization: functional decay resulting from the accumulation of mutations. According to the duplication-degeneration-complementation (DDC) model, duplicated genes are retained by subfunctionalization, where the functions of the ancestral gene are sub-divided between duplicate genes, or by neofunctionalization, where one of the duplicates acquires a new function. Here, we report the differential regulation of the zebrafish tandemly-duplicated fatty acid-binding protein genes, *fabp1b.1* and *fabp1b.2* by peroxisome proliferator-activated receptors (PPAR). *fabp1b.1* mRNA levels were induced in tissue explants of liver, but not intestine, by PPAR agonists. *fabp1b.1* promoter activity was induced to a greater extent by rosiglitazone (PPAR γ -selective agonist) compared to WY 14,643 (PPAR α -selective agonist) in HEK293A cells. Mutation of a peroxisome proliferator response element (PPRE) at -1,232 bp in the *fabp1b.1* promoter reduced PPAR-dependent activation. *fabp1b.2* promoter activity was not affected by PPAR agonists. Differential regulation of the duplicated *fabp1b* promoters may be the result of PPRE loss in *fabp1b.2* during a meiotic crossing-over event. Retention of PPAR inducibility in *fabp1b.1* and not *fabp1b.2* suggests unique regulation and function of the *fabp1b* duplicates.

Keywords

Duplication-degeneration-complementation; fatty acid-binding protein; gene duplication; peroxisome proliferator-activated receptor; promoter

Introduction

Gene duplication is thought to facilitate increasing organismal complexity by expanding gene function (Holland et al. 1994; Hurles 2004; Ohno 1970; Robinson-Rechavi et al. 2001; Sidow 1996). Gene duplication occurs by unequal crossing-over during meiosis, replication slippage, retrotransposition, aneuploidy, or whole genome duplication (Zhang 2003). According to the duplication-degeneration-complementation (DDC) model, the common fate of duplicated genes is nonfunctionalization, the loss of one duplicated gene copy owing to accumulated mutation and functional decay (Force et al. 1999; Lynch and Conery 2000). Both copies of a duplicated gene may, however, be retained in the genome if one of the duplicates acquires a novel function (neofunctionalization), or the functions of the ancestral gene are subdivided between the duplicates (subfunctionalization) (Force et al. 1999; Lynch and Conery 2000; Taylor and Rae, 2004). Altered function of duplicated genes occurs by either: mutation in the DNA of protein coding regions, or the loss or gain of *cis*-acting regulatory elements in the promoters of duplicated genes (Force et al. 1999; Lynch and Conery 2000; Taylor and Raes 2004). Changes in promoter regulatory elements may lead to differential control of tissue-, developmental stage- or stimulus-dependent transcription of duplicated genes (Force et al. 1999; Lynch and Conery 2000; Taylor and Raes 2004). Among studies of teleost evolution, mutations in protein coding regions, developmental stage- and tissue-specific gene expression, and the fate of duplicated genes have received greater attention compared to studies of defined specific differences in the *cis*-regulatory elements of duplicate gene promoters (Hurles 2004; Chan et al. 2010; Freeling et al. 2015). A growing body of evidence indicates that divergence of *cis*-regulatory elements in

duplicated genes affects phenotype, which in turn leads to natural selection of genotypes (Chan et al. 2010; reviewed in Wittkopp and Kalay 2012).

The zebrafish *fabp1b.1* and *fabp1b.2* genes are tandem duplicates (Fig. 1A). The tandem duplication of the *fabp1b* gene was subsequent to an earlier whole genome duplication, which gave rise to the duplicate genes, *fabp1a* and *fabp1b*, found in many teleost fish genomes (Glasauer and Neuhauss 2014). Thus far, tandem duplicates of *fabp1b* have only been found in the zebrafish genome. The gene structure of *fabp1b.1* [National Center for Biotechnology Information (NCBI), Gene ID: 554095] and *fabp1b.2* (NCBI, Gene ID: 100330224) is highly conserved, and consists of four exons separated by three introns (Fig. 1A). While the protein structure and amino acid sequence of zebrafish Fabp1b.1 and Fabp1b.2 are highly conserved, they differ in their developmental stage- and tissue-specific patterns of transcript distribution (summarized in Table 1) suggesting differentiation of *cis*-acting regulatory elements that control transcription of these genes (Brown et al. 2015; Karanth et al. 2009a; Sharma et al. 2006).

Peroxisome proliferator activated-receptors (PPAR) control transcription by binding PPAR response elements (PPRE) of PPAR target genes. The predominant role of PPAR α is as a regulator of fatty acid oxidation in the liver, kidney, small intestine, heart and skeletal muscle, while PPAR γ plays a critical role in adipocyte differentiation and lipid storage (Juge-Aubry et al. 1997). PPREs can favour binding of different PPAR isoforms (Hsu et al. 1998; Palmer et al. 1995). Binding of PPAR α and PPAR γ to the PPRE is dependent upon the direct repeat 1 (DR1) region of the PPRE (Hsu et al. 1998; Palmer et al. 1995). PPREs with high sequence similarity in the 5' flanking region (5'FR) to the consensus PPRE exhibit greater transcriptional activation by PPAR α than by

PPAR γ (Hsu et al. 1998; Juge-Aubry et al. 1997; Palmer et al. 1995).

Previous *in vivo* studies had implicated PPARs in regulation of the duplicated zebrafish *fabp1b* genes (Karanth et al. 2009a,b; Venkatachalam et al. 2012). The steady-state level of *fabp1b.1* mRNA increase in the intestine of fish fed a linolenic acid rich diet and in the heart of fish fed clofibrate, a non-selective partial PPAR agonist. In contrast, the steady-state level of *fabp1b.2* mRNA is unaffected by linolenic acid or clofibrate (Table 2) (Karanth et al. 2009b; Venkatachalam et al. 2012). We hypothesized that the differential regulation of the duplicated *fabp* genes by PPAR agonists may have arisen owing to loss or gain of a PPRE in one of the duplicated *fabp1b* genes. To test this hypothesis, we analysed: (1) *fabp1b.1* and *fabp1b.2* mRNA levels in zebrafish explant liver and intestine treated with PPAR agonists; (2) *fabp1b.1* and *fabp1b.2* promoters using *in silico* analysis to identify putative PPRES; and (3) *fabp1b.1* and *fabp1b.2* promoter activity in HEK293A cells transfected with *fabp1b.1* and *fabp1b.2* promoter fragments fused to the luciferase reporter gene and treated with PPAR agonists.

Materials and Methods

Retrieval of promoter sequences

Promoter sequences for zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* genes were obtained using the CNS Discovery Pipeline (v. 3.0) created and described by Turco et al. (Turco et al. 2013). The source code for the CNS Discovery Pipeline 3.0 is available for download at https://github.com/gturco/find_cns with instructions for installation at (https://github.com/gturco/find_cns/blob/master/INSTALL.rst) (Turco et al. 2013). CNS Discovery Pipeline was run using default settings except that the filter for promoter regions containing gene-coding regions was removed. The input was the zebrafish Zv9

whole genome assembly (GenBank Assembly ID GCA_000002035.2) (Benson et al. 2013). The resulting fasta output files for the *fabp* promoters and their corresponding genes were used for subsequent analysis and the design of PCR primers used to clone the *fabp* promoter fragments (File S1).

Transcription factor analysis

Promoter sequences were analyzed for putative PPREs using MatInspector (v. 8.1) with the Genomatix ElDorado genomes database and the vertebrates matrix group. The PPRE was defined as 5'-CAAACTAGGTCANAGGTCA-3' (Hsu et al. 1998; Palmer et al. 1995). The mismatch threshold was set to 35% (*i.e.* transcription factor sites were identified if they were 65% similar to the corresponding IUPAC string).

Primary cell culture from zebrafish liver and intestine explant tissue

Primary zebrafish cell culture methods were adapted from Kan et al. (2009). *Ex vivo* primary cell cultures of zebrafish liver and intestine were obtained from adult male fish. Fish were euthanized with tricaine (10% v/v) and rinsed with 70% ethanol in sterile phosphate-buffered saline (PBS). The liver and intestine were dissected, rinsed once with PBS, and incubated in 0.25% trypsin-EDTA (Gibco, Oakville, ON) for 5 min at room temperature. Tissue was suspended in trypsin-EDTA by pipette and then subjected to centrifugation at 500 $\times$ g for 5 min at room temperature. Cells were resuspended in media containing 50% Leibovitz's L-15, 35% high glucose DMEM, 15% Ham's F-12, 5% FBS, 0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL bovine insulin, and 50 ng/mL human EGF (Gibco) and maintained at 28°C, 100% atmospheric air on poly-D-lysine-coated cell culture plates. Primary zebrafish cells were maintained for 48 h prior to treatment with WY 14,643 or rosiglitazone (Ibabe et al. 2005). Zebrafish primary cells

from liver and intestine explant tissue were treated with either 1 μ M WY 14,643, 1 μ M rosiglitazone, or vehicle (0.5% DMSO). The concentration of these PPAR agonists was chosen based on previous studies (Lehmann et al. 1995; Keller et al. 1997). Media was changed daily. All protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care (CCAC). All animal protocols were approved by the Carleton Animal Care Committee at Dalhousie University prior to beginning the study.

Human embryonic kidney 293A cell culture

Human embryonic kidney 293A (HEK293A) cells were obtained from Cedarlane (Burlington, ON). HEK293A cells were maintained at 37°C, 5% CO₂ in DMEM containing 10% FBS and 10⁴ U/mL Pen/Strep. HEK293A cells express PPAR α and γ (Zagranichnaya et al. 2005) and this was confirmed by RT-PCR (data not shown).

*Cloning of zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* promoter fragments into pGL3-basic plasmid*

Zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* promoter sequences were amplified from genomic DNA by PCR. Genomic DNA was isolated from frozen liver using the GenElute Genomic DNA Miniprep kit according to the manufacturer's instructions (Sigma-Aldrich, Oakville, ON). PCR reagent concentrations were: 2 mM MgCl₂, 0.5 μ M forward and reverse primers (with DNA cassettes containing *Mlu*I and *Hind*III recognition sequences for direction cloning of promoters. See Table S1), 0.3 mM dNTPs, 1 U *Taq* DNA polymerase, and 40 ng genomic DNA. PCR conditions were: 95°C for 10 min; 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 6 min; and 72°C for 10 min. PCR products were resolved by gel electrophoresis and purified using the GenElute Gel Extraction kit (Sigma-Aldrich). Purified *acox1*, *fabp1b.1*, and *fabp1b.2* PCR products,

and the pGL3-Basic plasmid (Promega) were digested with *Mlu*I and *Hind*III according to the manufacturer's instructions (Fermentas, Burlington, ON). PCR products were ligated into pGL3-Basic at 16°C overnight using T4 DNA ligase according to the manufacturer's instructions (Invitrogen). IVNαF' electrocompetent *E. coli* (Invitrogen) were transformed with the constructed plasmids, *pacox1*, *pfabp1b.1*, and *pfabp1b.2*, propagated and plasmid DNA purified from the bacterial cells using the GenElute Plasmid Midiprep kit (Sigma-Aldrich). The pHRL-TK plasmid was obtained from Promega.

pfabp1b.1 mutant plasmids were generated by PCR-based site-directed mutagenesis (Papworth et al. 1996). Reactions were composed of 2 mM MgCl₂, 0.5 μM forward and reverse mutagenic primers (Table S1), 0.3 mM dNTPs, 1 U *Taq* DNA polymerase, and 40 ng plasmid DNA. PCR conditions were: 95°C 1 min, 18 cycles of 95°C 50 s, 60°C 1 min, and 68°C 8 min, followed by a final extension at 68°C for 10 min. Input plasmid was removed by digestion with the methylation-insensitive *Dpn*I (5 U) in 1X FastDigest Green Buffer® in a final volume of 20 μL (Fermentas) for 1 h at 37°C.

Transfection, treatment, and the dual luciferase assay in HEK293A cells

Transfections of HEK293A cells were performed using Lipofectamine 2000 reagent according to the manufacturer's instructions with 400 ng *pacox1*, *pfabp1b.1*, *pfabp1b.2*, or pGL3-Basic (background control) and 200 ng pHRL-TK (Invitrogen). The luciferase activity of the pHRL-TK plasmid containing the *Renilla* luciferase gene under the control of the cytomegalovirus thymidine kinase (TK) promoter was used to normalize firefly luciferase activity under the regulation of zebrafish promoters.

All drugs were obtained from Sigma-Aldrich. HEK293A cells were treated with either 100 nM WY 14,643 (PPAR α agonist), 100 nM rosiglitazone (PPAR γ agonist), or vehicle (0.5% DMSO). The concentration of PPAR agonists was chosen based on the EC₅₀ previously reported for WY 14,643 and rosiglitazone in mammalian cells (Keller et al., 1997; Lehmann et al. 1995), an EC₅₀ reproduced in HEK293A cells with the zebrafish *acox1* and *fabp1b.1* promoters in this study (data not shown). Luciferase activity was quantified by the dual luciferase according to the manufacturer's instructions (Promega).

Quantitative reverse transcriptase PCR

RNA was harvested from cells using the Trizol® (Invitrogen) extraction method according to the manufacturer's instruction. Reverse transcription reactions were carried out with SuperScript III® reverse transcriptase (+RT; Invitrogen), or without (-RT) as a negative control for use in subsequent PCR experiments according to the manufacturer's instructions. Two micrograms of RNA were used per RT reaction. qRT-PCR was conducted using the LightCycler® system and software (version 3.0; Roche, Laval, QC). Reactions were composed of a primer-specific concentration of MgCl₂ (Table S1), 0.5 μ M each of forward and reverse primers (Table S1), 2 μ L of LightCycler® FastStart Reaction Mix SYBR Green I, and 2 μ L cDNA to a final volume of 20 μ L with dH₂O (Roche). The PCR program was: 95°C for 10 min, 50 cycles of 95°C 10 s, a primer-specific annealing temperature (Table S1) for 5 s, and 72°C for 10 s. Experiments always included sample-matched -RT controls, a no-sample dH₂O control, and a standard control containing product-specific cDNA of a known concentration. cDNA abundance was calculated using the $\Delta\Delta$ CT method and normalized to GAPDH levels (Livak and Schmittgen 2001).

Statistical Analyses

Statistical analyses were conducted by one-way ANOVA followed by Tukey's *post-hoc* test or two-way ANOVA followed by Bonferroni's *post-hoc* test, as indicated. Homogeneity of variance was confirmed using Bartlett's test. The level of significance was set to $P < 0.001$, and all results are reported as the mean $\pm$ the standard error of the mean (SEM) from at least three independent experiments.

Results

To determine if transcriptional modulation of the *fabp1b.1* and *fabp1b.2* genes previously observed in adult zebrafish was PPAR-dependent (Karanth et al. 2009a,b; Venkatachalam et al. 2012), steady-state mRNA levels of the duplicated *fabp1b* genes were quantified in explant tissue derived from zebrafish liver and intestine. The steady-state level of acyl-CoA oxidase 1 (*acox1*) mRNA, a positive control for PPAR responsiveness, was detected in liver and intestine explants and induced in both tissues by either WY 14,643 (a PPAR α -selective agonist) or rosiglitazone (a PPAR γ -selective agonist) (Fig. 2A). *fabp1b.1* mRNA levels were increased 15-fold following WY 14,643 treatment, and 27-fold following rosiglitazone treatment in liver, but not in intestine explants (Fig. 2B). The steady-state level of *fabp1b.1* transcripts in liver explants was higher following rosiglitazone treatment than following WY 14,643 treatment (Fig. 2B), suggesting that *fabp1b.1* transcriptional induction was mediated by PPAR γ . The induction of *fabp1b.1* transcription by PPAR agonists is consistent with previous findings of Venkatachalam et al. (2012), who showed that induction of steady-state levels of *fabp1b.1* transcripts occurred in the liver, but not in the intestine of fish fed the non-selective PPAR agonist clofibrate. Catabolism of fatty acids in the intestine prior to

transport into the liver of adult zebrafish may explain why *fabp1b.1* mRNA levels were induced in liver explants following direct application of PPAR agonists, but were not induced in adult zebrafish liver (Karanth et al. 2009a). The steady-state levels of *fabp1b.2* mRNA transcripts in liver and intestine explants were not altered by PPAR agonist treatment, suggesting that transcription of the zebrafish *fabp1b.2* gene was not regulated by PPARs, which is consistent with the observation that the non-selective PPAR agonist clofibrate or fatty acid-enriched diets do not alter *fabp1b.2* levels (Karanth et al. 2009a; Venkatachalam et al. 2012).

To identify putative PPRES controlling transcription of the *fabp1b.1* and *fabp1b.2* genes, sequences of 3,059 bp and 3,218 bp 5' of the *fabp1b.1* and *fabp1b.2* transcription start sites (TSS), respectively, were retrieved using the CNS discovery pipeline (v. 3.0) (Turco et al. 2013) and putative PPRES identified using MatInspector (v. 8.1). Five putative PPRES were identified in the 3,059 bp promoter fragment of the *fabp1b.1* gene that exhibited 65%, 68.1%, 82.3% (indicated by the orange rectangle in Fig. 1), 65.1% and 76.2% sequence identity to the vertebrate PPRE consensus sequence (Hsu et al. 1997; Palmer et al. 1995). No PPRES were identified within the 3,218 bp sequence 5' of the TSS of the zebrafish *fabp1b.2* gene. The high sequence identity between the consensus PPRE and the putative PPRE at -1,232 bp in the *fabp1b.1* promoter suggested that the zebrafish *fabp1b.1* contained a functional PPRE. The lack of PPRES identified by *in silico* analysis of the *fabp1b.2* promoter was congruent with the lack of PPAR- and fatty acid-dependent transcriptional induction of *fabp1b.2* in whole fish and explant tissue (Fig. 2) (Karanth et al. 2009b; Venkatachalam et al. 2012).

To demonstrate that the *fabp1b.1* gene contained a functional PPRE and the *fabp1b.2* gene did not, promoter fragments 2,443 bp and 2,847 bp upstream of the *fabp1b.1* and *fabp1b.2* TSS, respectively, were PCR-amplified from zebrafish genomic DNA and cloned into the pGL3-Basic promoter-reporter plasmid, 5' to the firefly luciferase coding region for functional promoter assays. A 3,095 bp fragment of the zebrafish *acox1* promoter was similarly cloned and used as a positive control to assay for PPAR responsiveness (Palmer et al. 1995; Karanth et al. 2009a; Venkatachalam et al. 2012). All promoter fragments displayed similar basal promoter activity to the co-transfected TK promoter in HEK293A cells (data not shown). *acox1* promoter activity increased 72-fold following addition of the PPAR α -specific agonist, WY 14,643 (time to $\frac{1}{2}$ -maximum = 15 h), and 25-fold following addition of the PPAR γ -specific agonist, rosiglitazone (time to $\frac{1}{2}$ -maximum = 5 h), when assayed over a 24 h period (Fig. 3A). *fabp1b.1* promoter activity increased 17-fold following addition of the PPAR α -specific agonist, WY 14,643 (time to $\frac{1}{2}$ -maximum = 6 h), and 75-fold following addition of the PPAR γ -specific agonist, rosiglitazone (time to $\frac{1}{2}$ -maximum = 6 h), when measured over a 24 h period (Fig. 3B). *fabp1b.2* promoter activity was not changed by WY 14,643 or rosiglitazone in the 24 h period analyzed (Fig 3C). All promoters had achieved steady, maximal levels of activity by 24 h post-treatment (Fig. 3). These data suggested the *fabp1b.1* promoter contained a PPAR γ -selective PPRE accounting for its transcriptional activation by PPAR agonists, while the *fabp1b.2* promoter lacked PPRES and was not transcriptionally induced by PPAR agonists.

The PPRES sequence at -1,232 bp relative to the TSS in *fabp1b.1* displayed high sequence similarity to the PPRES consensus in the DR1 (5'-AGGTCANAGGTCA-3'), but

not 5'FR (5'-CAAAAC-3'). The 5'FR of the PPRE enhances the binding of PPAR α to the PPRE and therefore imparts greater PPAR α -selectivity to a PPRE (Juge-Aubry et al. 1997). PPRES with low sequence similarity in the 5'FR are more likely to be selective for PPAR γ compared to PPAR α (Hsu et al. 1997; Juge-Aubry et al. 1997; Palmer et al. 1995). Based on this observation, the putative PPRE identified at -1,232 bp was more likely PPAR γ -selective than PPAR α -selective. This predication was consistent with observations of the effects of PPAR α - and γ -selective agonists made in zebrafish explant liver cultures. Site-directed mutagenesis targeted at the putative *fabp1b.1* PPRE (-1,232 bp) was used to determine if this PPRE was indeed functional. Site-directed mutagenesis was performed on the 5'FR and the DR1 elements separately because the 5'FR has been shown to enhance PPAR α binding to PPRES whereas the DR1 is important for the general binding of PPAR (α or γ) to PPRES (Hsu et al. 1997; Juge-Aubry et al. 1997; Palmer et al. 1995). Mutagenesis of the 5'FR or DR1 of the *fabp1b.1* PPRE at -1,232 bp (Fig. 4A) resulted in decreased *fabp1b.1* promoter activity in cells treated with WY 14,643 compared to non-mutated *fabp1b.1* promoter activity in cells treated with WY 14,643 (Fig. 4B). Mutagenesis of the 5'FR of the *fabp1b.1* PPRE at -1,232 bp (Fig. 4A) did not change *fabp1b.1* promoter activity in cells treated with rosiglitazone, but mutagenesis of the DR1 of this PPRE significantly decreased *fabp1b.1* promoter activity in cells treated with rosiglitazone, compared to the activity of non-mutated (wild-type) *fabp1b.1* promoter activity in cells treated with rosiglitazone (Fig. 4B). Based on the activity of the wild-type, 5'FR, and DR1 mutant *fabp1b.1* promoter fragments, and their responsiveness to the PPAR agonists, WY 14,643 and rosiglitazone, transcriptional

induction of the zebrafish *fabp1b.1* gene appears to be predominantly controlled by a PPAR γ -selective PPRE at -1,232 bp upstream of the TSS (Fig. 4).

Steady-state *fabp1b.1* mRNA levels in zebrafish explant tissue and *fabp1b.1* promoter activity in HEK293A cells were induced to a greater extent by the PPAR γ -selective agonist, rosiglitazone, than the PPAR α -selective agonist WY 14,643. The PPRE at -1,232 bp was most likely the predominant site for PPAR-dependent transcriptional induction at the *fabp1b.1* promoter. Since the mutant *fabp1b.1* promoters retained some PPAR responsiveness to rosiglitazone, other PPREs must control PPAR-transcriptional induction at the *fabp1b.1* promoter. Previous studies have demonstrated the role of the 5'FR in enhancing the binding of PPAR α to the PPRE using non-quantitative electrophoretic mobility shift assays (Hsu et al. 1997; Juge-Aubry et al. 1997; Palmer et al. 1995; Wolfrum et al. 20001). The work presented here confirms the role of the 5'FR in PPAR α -dependent promoter activation, and the DR1 in general PPAR-dependent promoter activation, using quantifiable methods. Therefore, gene promoters (*e.g. fabp* gene promoters) are likely to be differentially selective to PPAR isoforms, which provides an additional layer of functionality to their regulation by PPARs.

Discussion

The data presented here demonstrate that *fabp1b.1* promoter activity was increased by PPAR agonists in both zebrafish explant tissue and in HEK293A cells transfected with a *fabp1b.1* promoter-reporter plasmid, whereas *fabp1b.2* promoter activity was unchanged by PPAR agonist treatment in explant tissue or HEK293A cells. WY 14,643 and rosiglitazone are well-characterized agonists of human PPAR (Den Broeder et al. 2015). WY 14,643 and rosiglitazone were agonists of human PPAR α and

PPAR γ that induced zebrafish *acox1* and *fabp1b.1* promoter activity in HEK293A cells. Zebrafish has two orthologs of human *PPARA* (*pparaa* and *pparab*) and one ortholog of human *PPARG* (*pparg*) (Den Broeder et al. 2015). In this study, WY 14,643 and rosiglitazone were agonists of zebrafish Ppar, as indicated by the agonist-dependent increase of *acox1* and *fabp1b.1* mRNA levels in zebrafish explant tissue (Fig. 2). WY 14,643 has been shown to activate zebrafish Ppara (Rizvi et al. 2013), but the specificity between Pparaa and Pparab is not known. Rosiglitazone displays significantly less potency and efficacy in HeLa cells expressing zebrafish Ppar γ compared to human PPAR γ (Riu et al. 2011; Grimaldi et al. 2015), suggesting the ligand binding domains of zebrafish and human PPAR γ may differ substantially. Although zebrafish and human PPAR are often considered to be structurally and functionally similar with respect to DNA binding and transcriptional regulation of target genes (Ibabe et al. 2005; Den Broeder et al. 2015), the greater diversity of PPAR genes in zebrafish than humans, and the differences in ligand binding, indicate a wider-range of possible responses to PPAR agonists in zebrafish compared to humans (Riu et al. 2011; Grimaldi et al. 2015). Given this greater diversity of PPAR response in zebrafish, PPAR isoform selectivity between zebrafish Pparaa, Pparab, and Ppar γ may differ from the selectivity observed between human PPAR α and PPAR γ at the *fabp1b.1* promoter in HEK293A cells. The data presented here demonstrate *fabp1b.1* promoter activity was PPAR γ -selective in the presence of zebrafish PPAR in zebrafish explant tissue (Fig. 2) and human PPAR in HEK293A cells (Figs. 3 and 4).

How did the PPAR-dependent regulation of the duplicated *fabp1b* genes of zebrafish diverge? The most parsimonious explanation is that the ancestral *fabp1b* gene

possessed a functional PPRE. Inspection of the nucleotide sequence of the *fabp1b.1* and *fabp1b.2* genes revealed long stretches of repetitive TAT sequences flanking both of the duplicated *fabp1b* genes (Fig. 1B). We propose, therefore, that tandem duplication of the *fabp1b* gene was the result of an unequal cross-over during meiosis, owing to misalignment of the repetitive TAT sequences that flanked the ancestral *fabp1b* gene. As a result, this highly active PPRE that resided 5' to the recombination site was deleted in the resulting extant chromosome 8, thereby leading to loss of transcriptional activation of the *fabp1b.2* gene by PPAR (Fig. 1B).

All vertebrate organisms possess multiple *fabp* genes with highly similar amino acid sequences and divergent developmental stage- and tissue-specific patterns of gene expression (Smather and Peterson 2011). These data provide evidence for increasing intra-organismal complexity – here, variation in an organism's response to fatty acids through PPAR stimuli – through the promoter regions of gene duplicates that have diverged from one another as each experienced differential selective pressure (Lynch and Conery 2000; Ohno 1970; Taylor and Raes 2004). Selective pressure against *fabp1b.1* or *fabp1b.2* is lacking because the existence of both genes represents a form of segregation avoidance such that two gene products sharing similar function are expressed in different tissues and conditions in all offspring (Conrad and Antonarakis 2007). While *Fabp1b.1* and *Fabp1b.2* sequences are relatively well conserved, exhibiting 75% amino acid similarity, ligand binding specificity and affinity for these Fabp proteins have yet to be determined. As such, substantial overlap in ligand specificity and affinity may exist between *Fabp1b.1* and *Fabp1b.2* (Karanth et al. 2009a). Consequently, the zebrafish *fabp1b.1* and *fabp1b.2* genes have undergone duplicate co-evolution (Hurles 2004). The

retention of PPAR-dependent inducibility in the *fabp1b.1* promoter and the loss of PPAR-dependent inducibility for the *fabp1b.2* promoter may contribute to the retention of these tandemly duplicated genes in the zebrafish genome (Lynch and Conery 2000; Smather and Peterson 2011; Taylor and Raes 2004; Zhang 2003). According to the DDC model as described in Force et al. (1999), the preservation of gene duplicates is more likely when mutations arise in the regulatory elements of duplicated genes that sub-divide gene functions compared to the ancestral gene. In the context of the DDC model, additional research is necessary to characterize differences in gene regulatory mechanisms besides the differential transcriptional control of these duplicated genes by PPAR, and their tissue-specific expression profiles (Sharma et al. 2003) that may account for the retention of *fabp1b.1* and *fabp1b.2* in the zebrafish genome through subfunctionalization of each paralog (Van Hellemont et al. 2007; Tong et al. 2012; Freeling et al. 2015). Further characterization of additional *fabp* promoters may help to define the evolutionary history of *fabp* genes that led to the retention of 57% of duplicate *fabp* genes in the zebrafish genome compared to only ~3% of all duplicated genes in the zebrafish genome following the teleost-specific whole genome duplication event (Woods et al. 2005; Guo et al. 2011).

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Tables

Table 1. Basal *acox1*, *fabp1b.1*, and *fabp1b.2* mRNA levels in tissues of embryo, larva, and adult zebrafish.

Gene	Life Stage	Tissue											
		Intestine	Liver	Heart	Brain	Kidney	Muscle	Ovary	Testis	Eye	Skin	SB	Gills
<i>acox1</i>	Larva	+	+	-	+	-	-	-	-	+	-	-	-
	Adult	+	+	+	+	+	+	+	+	-	-	-	-
<i>fabp1b.1</i>	Larva	+	-	-	-	-	-	-	-	-	-	-	-
	Adult	+	+	+	+	+	+	+	+	+	+	-	+
<i>fabp1b.2</i>	Larva	-	-	-	+	-	-	-	-	-	-	-	-
	Adult	+	+	+	+	-	-	+	+	+	+	-	+

+ mRNA transcript detectable at basal levels, - not detected

Data are a summary of previous findings from Howe et al. 2013 (ZFIN database: *acox1*, *fabp1b.1*, *fabp1b.2*); Karanth et al. 2009a,b; and Venkatachalam et al. 2012.

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Table 2. Inducible *acox1*, *fabp1b.1* and *fabp1b.2* mRNA levels in tissues of adult zebrafish fed clofibrate or linolenic acid.

Gene	Tissue					
	Intestine	Liver	Heart	Brain	Kidney	Muscle
<i>acox1</i>	C+	C+	C+	NA	C+	C+
<i>fabp1b.1</i>	L+	NA	C+	NA	NA	NA
<i>fabp1b.2</i>	NA	NA	NA	NA	–	–

C+, induced by clofibrate diet; L+, induced by linolenic acid diet;
NA, expressed but not affected, not expressed.

Data are a summary of previous findings from Karanth et al. 2009a,b; and Venkatachalam et al. 2012.

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Figure captions

Figure 1. Genomic organization and tandem duplication of the zebrafish *fabp1b.1*

and *fabp1b.2* genes. (A) *fabp1b.1*, and *fabp1b.2* are each flanked by TAT repeat elements of varying lengths. (B) Misalignment of neighbouring TAT elements on chromosome 8 during meiosis generated the tandem gene duplication of the *fabp1b.1* and *fabp1b.2* genes (NC_007116 Ch. 5 and NC_007119.5 Ch. 8 Ref Zv9 Primary Genome Assembly). Repetitive TAT elements are indicated by blue boxes with the number of repeats written in brackets below each box. The TSS for each gene coding region is indicated by a black arrow. *fabp1b.1* and *fabp1b.2* exons (1-4) are indicated by grey boxes. Scale of the genome assembly is 15 kb from the first exon of *fabp1b.1* to the fourth exon of *fabp1b.2*. A putative PPRE shown in orange with 82.3% sequence identity to the vertebrate PPRE consensus sequence was identified by MatInspector (v. 8.1). No PPRES were identified in the 5' upstream region of the *fabp1b.2* gene.

Figure 2. Tissue-specificity of the PPAR-dependent induction of steady-state levels of zebrafish *fabp1b.1* mRNA transcripts. Primary cells derived from zebrafish liver and intestine explant tissue were cultured *ex vivo* for 48 h before treatment with either 1 μ M WY 14,643 (PPAR α -selective agonist) or 1 μ M rosiglitazone (PPAR γ -selective agonist) for 24 h. Steady-state levels of (A) *acox1*, (B) *fabp1b.1*, and (C) *fabp1b.2* mRNA transcripts were quantified by qRT-PCR using the $\Delta\Delta$ CT method and normalized to GAPDH. * $P < 0.001$ compared to vehicle treatment within tissue, † $P < 0.001$ compared to WY 14,643 treatment within tissue as determined *via* two-way ANOVA followed by Bonferroni's *post-hoc* test. $n = 4$.

Figure 3. Differential induction of zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* promoter activity by isoform-specific PPAR agonists. Firefly luciferase activity under the regulatory control of the (A) *acox1*, (B) *fabp1b.1*, or (C) *fabp1b.2* promoters was normalized to *Renilla* luciferase activity driven by the TK promoter in HEK293A cells treated with either 100 nM WY 14,643 (PPAR α -selective agonist) or 100 nM rosiglitazone (PPAR γ -selective agonist) over a 24 h period. * $P < 0.001$ compared to other drug within time point as determined *via* one-way ANOVA followed by Tukey's *post-hoc* test. $n = 3$.

Figure 4. The PPRE at -1,232 bp was the major regulatory site for PPAR induction of zebrafish *fabp1b.1* promoter activity. (A) Site-directed mutagenesis of the PPAR binding site at -1,232 bp (relative to TSS indicated by arrow) in the *fabp1b.1* promoter fragment. Each segment of *fabp1b.1* promoter represents 500 bp. (B) Firefly luciferase activity under the regulatory control of the wild-type (*fabp1b.1*) (**black bar**), 5' flanking region mutant (*fabp1b.1* Δ 5'FR) (**blue bar**), or direct repeat element mutant (*fabp1b.1* Δ DR1) (**purple bar**) promoters was normalized to *Renilla* luciferase activity driven by the TK promoter in HEK293A cells treated with either 100 nM WY 14,643 (PPAR α -selective agonist) or 100 nM rosiglitazone (PPAR γ -selective agonist) for 24 h. * $P < 0.001$ compared to *fabp1b.1*/TK (**black bar**), ^ $P < 0.001$ compared to *fabp1b.1* Δ 5'FR/TK (**blue bar**) within drug as determined *via* two-way ANOVA followed by Bonferroni's *post-hoc* test. $n = 3$.

Figure 1. Genomic organization and tandem duplication of the zebrafish *fabp1b.1* and *fabp1b.2* genes. (A) *fabp1b.1*, and *fabp1b.2* are each flanked by TAT repeat elements of varying lengths. (B) Misalignment of neighbouring TAT elements on chromosome 8 during meiosis generated the tandem gene duplication of the *fabp1b.1* and *fabp1b.2* genes (NC_007116 Ch. 5 and NC_007119.5 Ch. 8 Ref Zv9 Primary Genome Assembly). Repetitive TAT elements are indicated by blue boxes with the number of repeats written in brackets below each box. The TSS for each gene coding region is indicated by a black arrow. *fabp1b.1* and *fabp1b.2* exons (1-4) are indicated by grey boxes. Scale of the genome assembly is 15 kb from the first exon of *fabp1b.1* to the fourth exon of *fabp1b.2*. A putative PPRE shown in orange with 82.3% sequence identity to the vertebrate PPRE consensus sequence was identified by MatInspector (v. 8.1). No PPRES were identified in the 5' upstream region of the *fabp1b.2* gene.

254x190mm (144 x 144 DPI)

Figure 2. Tissue-specificity of the PPAR-dependent induction of steady-state levels of zebrafish *fabp1b.1* mRNA transcripts. Primary cells derived from zebrafish liver and intestine explant tissue were cultured *ex vivo* for 48 h before treatment with either 1 μM WY 14,643 (PPARα-selective agonist) or 1 μM rosiglitazone (PPARγ-selective agonist) for 24 h. Steady-state levels of **(A)** *acoxy1*, **(B)** *fabp1b.1*, and **(C)** *fabp1b.2* mRNA transcripts were quantified by qRT-PCR using the $\Delta\Delta C_T$ method and normalized to GAPDH. **P* < 0.001 compared to vehicle treatment within tissue, †*P* < 0.001 compared to WY 14,643 treatment within tissue as determined *via* two-way ANOVA followed by Bonferroni's *post-hoc* test. *n* = 4.

Figure 3. Differential induction of zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* promoter activity by isoform-specific PPAR agonists. Firefly luciferase activity under the regulatory control of the (A) *acox1*, (B) *fabp1b.1*, or (C) *fabp1b.2* promoters was normalized to *Renilla* luciferase activity driven by the TK promoter in HEK293A cells treated with either 100 nM WY 14,643 (PPAR α -selective agonist) or 100 nM rosiglitazone (PPAR γ -selective agonist) over a 24 h period. * $P < 0.001$ compared to other drug within time point as determined via one-way ANOVA followed by Tukey's *post-hoc* test. $n = 3$. 93x179mm (300 x 300 DPI)

Figure 4. The PPRE at -1,232 bp was the major regulatory site for PPAR induction of zebrafish *fabp1b.1* promoter activity. (A) Site-directed mutagenesis of the PPAR binding site at -1,232 bp (relative to TSS indicated by arrow) in the *fabp1b.1* promoter fragment. Each segment of *fabp1b.1* promoter represents 500 bp. (B) Firefly luciferase activity under the regulatory control of the wild-type (*fabp1b.1*) (black bar), 5' flanking region mutant (*fabp1b.1* Δ5'FR) (blue bar), or direct repeat element mutant (*fabp1b.1* ΔDR1) (purple bar) promoters was normalized to *Renilla* luciferase activity driven by the TK promoter in HEK293A cells treated with either 100 nM WY 14,643 (PPARα-selective agonist) or 100 nM rosiglitazone (PPARγ-selective agonist) for 24 h. **P* < 0.001 compared to *fabp1b.1*TK (black bar), ^*P* < 0.001 compared to *fabp1b.1* Δ5'FR/TK (blue bar) within drug as determined *via* two-way ANOVA followed by Bonferroni's *post-hoc* test. *n* = 3.

Supplementary file S1. Conserved non-coding (promoter) sequence files (.fasta) for zebrafish *acox1*, *fabplb.1*, and *fabplb.2* were determined from sequencing of PCR-cloned promoter fragments of genomic DNA (this study). Putative PPREs are indicated as green 65 – 74.9%, yellow 75 – 84.9%, and orange > 85% sequence identity to the defined vertebrate PPRE consensus sequence. Primer-binding sites for PCR cloning of promoter fragments are highlighted in red and repetitive TAT sequences are highlighted in light blue (see Fig. 1).

>*acox1*

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ATTTCTCACAACCCTATTTACATTTGCACAACAGTTAATGCATTTCTCAAAACAATTAGTCATT
TGTACACATCATAGTAGCAGTTTCTCATTTGCTTCCAACAAATTGCAAATACCTGTGGACATGCA
TTAATTGCTTTTCATACAACCTCTCTGCTGTTTTATAACATTATTGTTTGTATGTCATGTCAGG
CAAAATTAATACTAACTTGTGAATGCTGAAAAGTCATTCCATATAAACTAATAGTCCTAATTTTC
ATTACTTGAGTCATTCCATACAAAAATGTTAACTAGTTGTCAAAATCTGTCAAGCTAATTTTC
TAAAAAATAATTTTTTTTTTCCAGAAAATGTCTCCTAGATTGAACAATTTTCATTAATGATTTAC
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[illegible]

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CATTGATGTTGAGTGTAT

Table S1. Synthetic oligonucleotides used in this study for cloning zebrafish *acox1*, *fabp1b.1*, and *fabp1b.2* promoter fragments, mutagenesis of the *fabp1b.1* PPRE, and quantification of *acox1*, *fabp1b.1*, and *fabp1b.2* transcripts by qRT-PCR in this study.

Target*	Sequence (5' - 3')†	Product Length (bp)	[MgCl ₂] (mM)	Annealing Temp. (°C)
<i>acox1</i>	ACGCGTTTCTTTTGCACAACGCTGTAA AAGCTTTGGTAACATTTGACGTCAAT	2481	2	57
<i>fabp1b.1</i>	ACGCGTCCAGGCCATATCCTGAGCTG AAGCTTTCCCAACACTGGGAAACACC	2443	2	57
<i>fabp1b.2</i>	ACGCGTACAACATGCTGGAACGGGAA AAGCTTTCTCACAACACTACTGATGCCCTT	2847	2	57
<i>fabp1b.1</i> Δ5'	TGGTTCGAGTTCGGGTTACGATAAG CTTATCCGTAACCGAACTCGAACCA	-	2	68
<i>fabp1b.1</i> ΔDR1	AAACTACGGACGGAACCATGAATTGG CCAATTCATGGTTCGGTCCGTAGTTT	-	2	68
<i>acox1</i> qRT-PCR	GTGTACTGCTGAACAACGCA CACGGATGTGTGTACCGTGC	104	3	59
<i>fabp1b.1</i> qRT-PCR	AACTGGTCAACACTCTGACG AAGTCTTGCGTGTGTTTGCT	98	1	58
<i>fabp1b.2</i> qRT-PCR	ATCCAGATGCTGAGCGGAGA ATGTTGTCAGCGGTGCTCAG	146	2	60
<i>GAPDH</i> qRT-PCR	GGCCGTTACAAGGGAGAAGT GTGGACTCGACCACGTACAG	137	2	59

*SDM, primers used in site-directed mutagenesis

†Bold font denotes *Mlu*I (Fwd) and *Hind*III (Rev) restriction sites, underlined nucleotides denote mutagenized residues.