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Spatiotemporal expression of carnitine palmitoyltransferase I genes during zebrafish development and heart regeneration

WENPING HUANG, CHANG KONG, XIAOHAN CHENG, ZONGYI DUAN, HAO CAO, YANCHAO HAN*

Institute for Cardiovascular Science and Department of Cardiovascular Surgery of the First Affiliated Hospital of Soochow University, Suzhou Medical College, Soochow University, Suzhou, Jiangsu, P. R. China

ABSTRACT Carnitine palmitoyltransferase 1 (CPT1) is a key regulatory enzyme in fatty acid metabolism, responsible for the translocation of long-chain fatty acids into the mitochondria for β-oxidation in diverse biological contexts. Recent studies implicated the critical role of *cpt1* genes during zebrafish development and heart regeneration; however, a comprehensive characterization of their spatiotemporal expression dynamics remains lacking. Here, we systematically analyzed the expression profiles of four *cpt1* paralogs (*cpt1aa*, *cpt1ab*, *cpt1b*, and *cpt1a2b*) during zebrafish embryogenesis and the expression of *cpt1ab* and *cpt1b* during zebrafish heart regeneration. Our results reveal that these paralogs exhibit distinct spatiotemporal expression patterns during zygotic development. While *cpt1aa* and *cpt1ab* share high sequence conservation (77%), their expression patterns diverge substantially. Conversely, *cpt1ab* and *cpt1b* display convergent cardiac and somitic expression despite lower sequence similarity (53%). Following ventricular ablation, *cpt1b* expression transiently ceased then recovered during regeneration, whereas *cpt1ab* remained unchanged. These findings shed light on the evolutionary conservation and functional divergence of *cpt1* paralogs, which establish a critical foundation for elucidating paralog-specific roles in fatty acid metabolism during vertebrate development and regeneration.

KEYWORDS: carnitine palmitoyltransferase, cpt1, development, heart regeneration, expression, zebrafish

Introduction

Fatty acid metabolism sustains energy homeostasis, membrane biogenesis and signal transduction. Carnitine palmitoyltransferase 1 (Cpt1), a key rate-limiting enzyme in fatty acid beta-oxidation, facilitates the transport of long-chain fatty acids into mitochondria for energy metabolism (Schlaepfer and Joshi, 2020). The mammalian CPT1 gene family consists of three paralogs: *Cpt1a*, *Cpt1b*, and *Cpt1c*, each exhibiting distinct tissue distributions, enzymatic activities and regulatory mechanisms (Wang *et al*., 2021). Early studies have shown that human and/or mouse *CPT1A* is predominantly expressed in the liver, *CPT1B* in muscle tissues, and *CPT1C* in the brain (Britton *et al*., 1995; Yamazaki, 2004; Price *et al*., 2002; Wolfgang *et al*., 2006). The expression of *Cpt1* genes is highly regulated under various physiological and pathological conditions. For instance, *Cpt1b* expression is transiently increased after birth (Bartelds *et al*., 2004; Brown *et al*., 1995; Lavrentyev *et al*., 2004) in rats and lambs, and can be induced by exercise or high fructose diet (Shen *et al*., 2015; Melendez-Salcido *et al*., 2025) in rats and mice, suggesting that *Cpt1* plays a crucial role in the regulatory response to metabolic stress. As a pivotal regulatory enzyme in fatty acid metabolism, *Cpt1* is not only closely associated with metabolic diseases such as obesity, diabetes, and fatty liver, but also widely involved in the development, growth and heart regeneration (Cao *et al*., 2019; Li *et al*., 2023; Ji *et al*., 2008). Nevertheless, the studies of the expression and roles of *Cpt1* genes in early development remain relatively scarce.

Zebrafish, as an important model organism, has a highly conserved early developmental process with mammals, providing an ideal model for studying gene function and developmental regulation. Previous phylogenetic analyses identified four *cpt1* genes from zebrafish genome—*cpt1aa*, *cpt1ab*, *cpt1b*, and *cpt1a2b* (Morash *et al*., 2010; Lopes-Marques *et al*., 2015). Among these, *cpt1aa* and *cpt1ab*, generated after the teleost-specific genome duplication (TGD), are orthologs of mammalian *Cpt1a*,

***Address correspondence to:** Yanchao Han. Institute for Cardiovascular Science and Department of Cardiovascular Surgery of the First Affiliated Hospital of Soochow University, Suzhou Medical College, Soochow University, Suzhou, Jiangsu 215000, P. R. China. E-mail: ychan@suda.edu.cn | https://orcid.org/0000-0002-1838-0836

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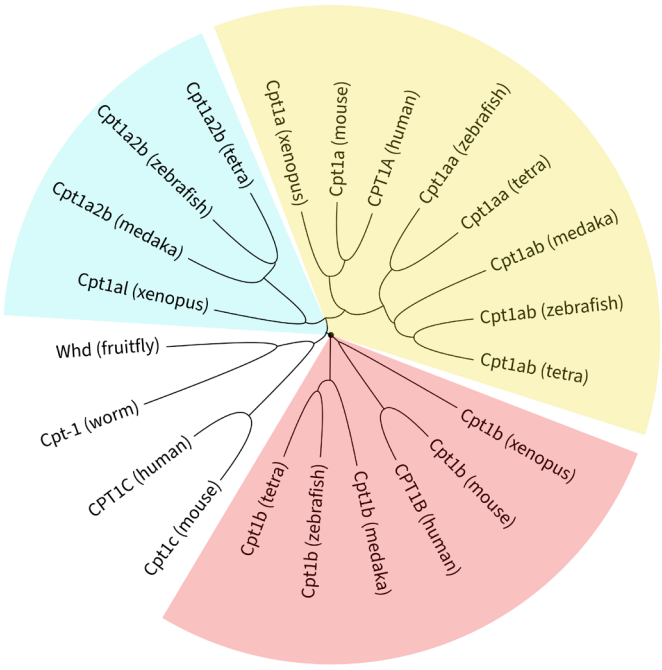


Fig. 1. Molecular phylogenetic analysis of the carnitine palmitoyltransferase 1 (CPT1) gene family. Evolutionary relationships of 22 CPT1 protein sequences across nine species. See Supplementary Data 1 for sequence accessions. Multiple sequence alignment was performed using ClustalX 2.1 and the resulting phylogeny was visualized and annotated using Interactive Tree of Life (iTOL v7).

and *cpt1b* is the ortholog of mammalian *Cpt1b*, while the orthologous relationship of *cpt1a2b* gene remains controversial (Morash *et al*., 2010; Lopes-Marques *et al*., 2015). Conservation analysis demonstrates higher protein sequence similarity between *cpt1a2b* and human *CPT1A*, whereas synteny analysis supports potential *cpt1a2b* orthology with human *CPT1C* (Lopes-Marques *et al*., 2015).

Recently, zebrafish have been adopted to study the role of *cpt1* genes in energy homeostasis, early development and heart regeneration. For instance, Li *et al.,* found that knocking out *cpt1b* in zebrafish reduced fatty acid oxidation and induced energy homeostasis remodeling in the liver and muscle (Li *et al*., 2020); Zecchin *et al.,* and Ulhaq *et al.,* reported that *cpt1a* knockdown impaired cilia growth, photoreceptor cell and lymphatics development (Ulhaq *et al*., 2023; Zecchin *et al*., 2018); we and Zhao *et al.,* found that fatty acid oxidation and *cpt1b* are essential for cardiomyocyte proliferation and ventricle regeneration (Zhao *et al*., 2024; Cheng *et al*., 2024). These studies highlight the rising significance of characterizing the role and mechanism of *cpt1* genes in zebrafish embryos. Despite this, the spatial-temporal expression of zebrafish *cpt1* genes has not yet been well studied.

In this study, we systematically profile the spatiotemporal expression of zebrafish *cpt1* genes throughout early development and during heart regeneration using *in situ* hybridization technique, which will provide important experimental evidence to understand the role of *cpt1* genes and fatty acid metabolism during embryonic development and heart regeneration.

Results

Phylogenetic analysis of CPT1 gene family in evolution

To comprehensively characterize the CPT1 gene family in zebrafish, we systematically queried the Zebrafish Information Network (ZFIN) database, and identified five putative paralogs: *cpt1aa*, *cpt1ab*, *cpt1b*, *cpt1a2a*, and *cpt1a2b*. The *cpt1a2a* locus was excluded from subsequent studies due to its incomplete sequence (<500 nt) lacking discernible protein domains. For evolutionary conservation assessment, we retrieved CPT1 protein sequences from three teleost species—zebrafish (*D. rerio*), medaka (*O. latipes*) and Mexican tetra (*A. mexicanus*)—as well as five additional species representing major phylogenetic lineages: worm (*C. elegans*), fruitfly (*D. melanogaster*), *Xenopus* (*X. tropicalis*), mouse (*M. musculus*), and human (*H. sapiens*).

Consistent with previous studies (Morash *et al*., 2010; Lopes-Marques *et al*., 2015), teleost *cpt1aa* and *cpt1ab* represent co-orthologs of mammalian *CPT1A* originating from TGD, although only *cpt1ab* has been identified in the medaka genome (Fig. 1). Pairwise analysis reveals that zebrafish Cpt1aa and Cpt1ab share 71% and 72% amino acid identity with human CPT1A, respectively. Similarly, teleost *cpt1b* maintains direct orthology with mammalian *CPT1B*, with 65% amino acid identity between zebrafish Cpt1b and human CPT1B. The paralog *cpt1a2b* (formerly designated *cpt1cb*) exhibits greater evolutionary similarity with CPT1A than CPT1C, and zebrafish Cpt1a2b shares 66% identity with human CPT1A versus 53% with CPT1C. This phylogenetic positioning suggests functional divergence following genome duplication events.

Spatiotemporal expression pattern of *cpt1aa* in zebrafish embryos

To systematically investigate the expression profile of *cpt1aa* gene during zebrafish embryonic development, whole-mount *in situ* hybridization (WISH) was performed using a *cpt1aa*-specific riboprobe across multiple critical developmental stages. As illustrated in Fig. 2, ubiquitous distribution of *cpt1aa* transcripts persists from 1-cell stage through bud stage (Fig. 2 A-D), indicating that *cpt1aa* is maternally expressed and maintains comparable expression beyond zygotic genome activation (ZGA). This pan-embryonic pattern undergoes progressive spatial restriction post-bud stage, with *ctp1aa* transcripts becoming enriched in the developing nervous system by 24 hours post-fertilization (hpf; Fig. 2E). By 2 days post-fertilization (dpf), *cpt1aa* exhibits differential neural compartmentalization, with strong signals persist in the brain, whereas spinal cord expression becomes undetectable (Fig. 2F).

At 3 dpf, concurrent with sustained *cpt1aa* expression in the developing brain, *cpt1aa* transcripts are robustly detected in the nascent hepatic bud (Fig. 2G), indicating early involvement of Cpt1aa-mediated β-oxidation during hepatic specification and metabolic maturation. Subsequent developmental progression reveals a complete neural-to-hepatic transition of *cpt1aa* expression starting from 4 to 5 dpf: brain signals become undetectable, whereas robust expression is maintained in the developing liver (Fig. 2 H-I). This spatiotemporal trajectory indicates that zebrafish *cpt1aa*, akin to mammalian *Cpt1a*, is predominantly expressed in the liver, although its widespread expression prior to liver morphogenesis suggests potential multifunctional roles beyond hepatic metabolism during early embryogenesis.

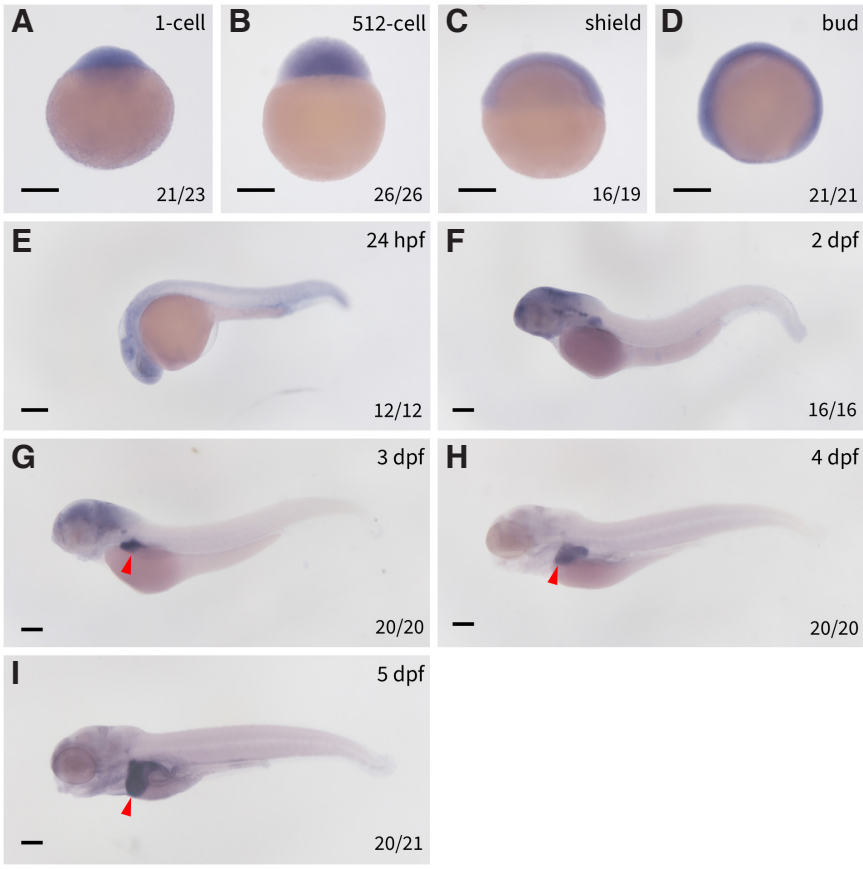


Fig. 2. Spatiotemporal expression of *cpt1aa* in zebrafish embryos. Expression of *cpt1aa* in **(A)** 1-cell stage embryos, **(B)** 512-cell embryos, **(C)** shield stage embryos, **(D)** bud stage embryos, **(E)** 24 hpf embryos, **(F)** 2 dpf embryos, **(G)** 3 dpf embryos, **(H)** 4 dpf embryos, **(I)** 5 dpf embryos. The red triangles denote the livers. dpf, days post fertilization; hpf, hours post fertilization; scale bars: 200 μm.

Divergent expression of *cpt1ab* following teleost genome duplication

Although *cpt1ab* shares ancestral orthology with *cpt1aa*, WISH analysis reveals distinct expression patterns that underscore their functional divergence. While maternal *cpt1ab* transcripts persist from 1-cell to 512-cell stages (Fig. 3 A-B), its expression vanishes from shield to bud stages (Fig. 3 C-D), suggesting complete degradation of maternal transcripts during gastrulation, which is contrast to the sustained post-ZGA expression observed for *cpt1aa*.

De novo zygotic *cpt1ab* expression remain undetectable until 24 hpf, when transcripts are simultaneously detected in cardiac primordium and somitic musculature through 2 dpf (Fig. 3 E-F). Extensive tissue-specific modulation occurs from 3 dpf onward: cardiac expression persists robustly from 24 hpf to 5 dpf (Fig. 3 F-I), whereas somitic signals diminish significantly by 4 dpf (Fig. 3H). Concurrently, endodermal activation commences at 3 dpf in liver and intestinal precursors (Fig. 3G), which is significantly elevated at 4 and 5 dpf (Fig. 3 H-I), with pharyngeal arch expression emerging at 5 dpf (Fig. 3I). This biphasic expression kinetics—marked by early cardiac-somitic predominance followed by endodermal expansion—contrasts sharply with *cpt1aa*'s hepatic commitment. Such divergence illustrates functional specialization following TGD, potentially facilitating metabolic adaptation in rapidly contracting tissues during early development, while supporting endodermal organ maturation in later stages.

Conserved muscular expression of *cpt1b* in zebrafish embryos

Similar with *cpt1ab*, zebrafish *cpt1b* exhibits maternally deposited transcripts at 1-cell and 512-cell stages (Fig. 4 A-B), followed by post-ZGA degradation, as evidenced by diminished expression from shield to bud stages (Fig. 4 C-D). Zygotic *ctp1b* expression initiates at 24 hpf with transcripts detectable in developing myotomes (Fig. 4E). Cardiac expression emerges at 2 dpf with ventricular predominance by 3 dpf (Fig. 4 F-I), which is consistent with chamber-specific metabolic demands of the heart. Notably, strong *cpt1b* transcripts are transiently expressed in endodermal tissues, including the intestine and pharyngeal pouches (Fig. 4H), suggesting a transient adaption for enteric lipid metabolism during early development. The cardiac and muscular expression of *cpt1b* is consistent with previous studies showing that CPT1B is mainly expressed in muscular tissues and plays a predominant role in high-energy-demand tissues.

Spatially restricted expression of *cpt1a2b* in zebrafish embryos

Similar to other *cpt1* paralogs, *cpt1a2b* exhibits conserved maternal transcript deposition (Fig. 5 A-B), yet exhibits distinct post-ZGA dynamics. While *cpt1aa* maintains a relatively stable expression level post-ZGA, and *cpt1ab* and *cpt1b* transcripts are almost completely degraded after this transition, *cpt1a2b* maintains low-level ubiquitous expression throughout gastrulation (shield to bud stages; Fig. 5 C-D). At the bud stage, a striking and specific upregulation of *cpt1a2b* expression emerges specifically within the prechordal plate mesendoderm—specifically in the polster cells (Fig. 5D, arrow), which is a critical domain for craniofacial organizer and absent in other paralogs.

cpt1a2b transcripts become restricted to the posterior trunk by 24 hpf (Fig. 5E), localizes exclusively to the pectoral fin bud by 2 dpf (Fig. 5F), suggesting potential roles in appendicular morphogenesis. Subsequent pharyngeal endoderm enrichment becomes evident from 3 dpf onward (Fig. 5 G-H). The progressively constrained spatiotemporal expression pattern of *cpt1a2b* suggesting that it may have a more specialized role in diverse developmental processes.

Differential expression of *cpt1ab* and *cpt1b* during heart regeneration

Zebrafish possess remarkable cardiac regenerative capacity, making them a valuable model for deciphering heart repair mechanisms. Previous studies established the *Tg(vmhc:mCherry-NTR)* zebrafish line for chemogenetic ventricular ablation, wherein the ventricular-specific myosin heavy chain (*vmhc*) promoter drives expression of a mCherry-nitroreductase (NTR) fusion pro-

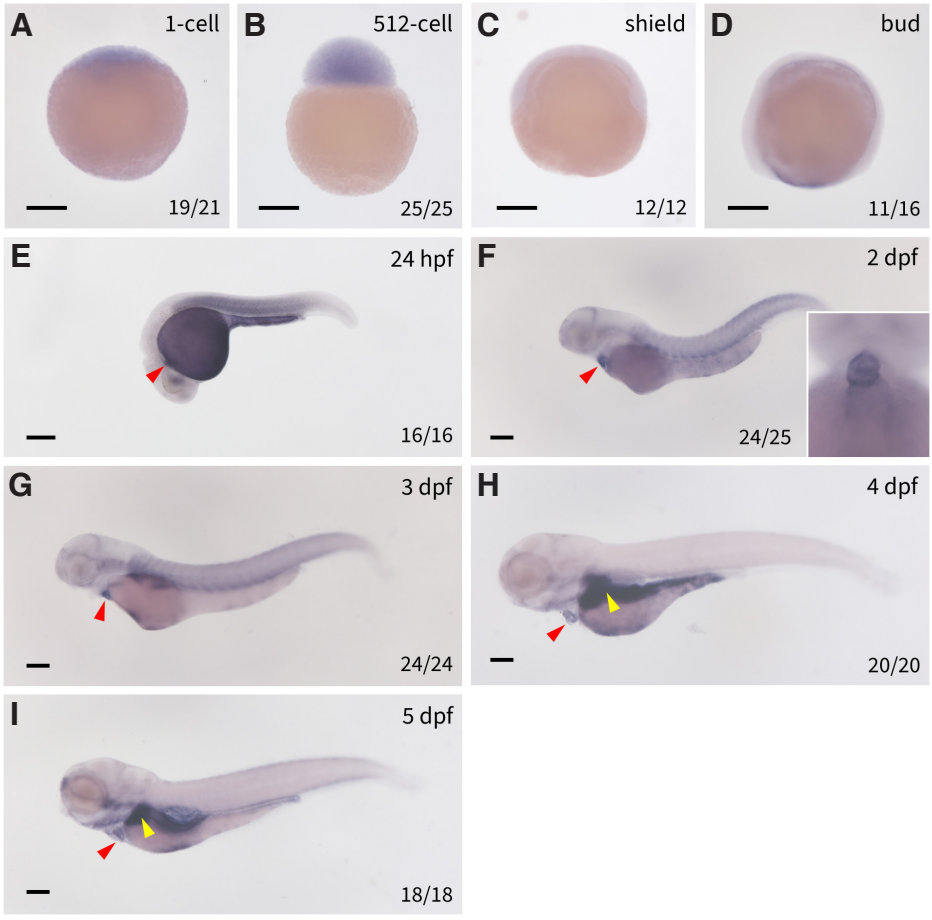


Fig. 3. Spatiotemporal expression of *cpt1ab* in zebrafish embryos. Expression of *cpt1ab* in **(A)** 1-cell stage embryos, **(B)** 512-cell embryos, **(C)** shield stage embryos, **(D)** bud stage embryos, **(E)** 24 hpf embryos, **(F)** 2 dpf embryos (the inset is a ventral view of the embryo showing the expression of *cpt1ab* in the heart), **(G)** 3 dpf embryos, **(H)** 4 dpf embryos, **(I)** 5 dpf embryos. The red triangles denote the hearts, and the yellow triangles denote the liver and intestine. Scale bars: 200 μm.

tein to enable metronidazole (MTZ)-dependent cardiomyocyte ablation via prodrug conversion (Zhang *et al*., 2013). Ronidazole (RDZ) has been reported to induce target cell ablation at lower concentrations with reduced toxicity compared to MTZ (Lai *et al*., 2021). To validate RDZ as a suitable ablation prodrug for zebrafish hearts, we crossed the *Tg(vmhc:mCherry-NTR)* zebrafish to *Tg(myl7:EGFP)* zebrafish which expresses EGFP in all cardiomyocytes, and treated their offspring with RDZ since 36 hpf. RDZ treatment significantly reduced ventricular size and induced severe pericardial edema exclusively in *Tg(vmhc:mCherry-NTR;myl7:EGFP)* embryos, but not in *Tg(myl7:EGFP)* controls (Fig. 6A), confirming effective induction of ventricular cardiomyocyte ablation with RDZ.

Given the cardiac expression of *cpt1ab* and *cpt1b* during development, we investigated their expression dynamics during heart regeneration. As shown in Fig. 6B, *cpt1ab* expression persisted post-ablation with no visible difference between DMSO- and RDZ-treated embryos, indicating minimal responsiveness to ventricular damage. In contrast, *cpt1b* expression was completely abolished after ablation, then gradually recovered from 1 day post-treatment (dpt) onward, reaching levels comparable to DMSO controls by 3 dpt (Fig. 6C). These data demonstrate that *cpt1b*, but not *cpt1ab*, exhibits dynamic expression changes in response to ventricular ablation, suggesting a potential role for *cpt1b* in zebrafish heart regeneration.

Discussion

This study presents the first comprehensive spatiotemporal expression study of the four *cpt1* paralogs during zebrafish embryogenesis. While zygotic expression diverges markedly among these paralogs, their universal maternal expression indicates an indispensable role of *cpt1* genes and fatty acid oxidation (FAO) in oogenesis and early developmental processes. This hypothesis aligns with extensive evidence demonstrating that pharmacological inhibition of FAO disrupts mouse oocyte maturation, early cleavage and blastulation, whereas enhanced FAO capacity improves mouse and yellow catfish oocyte quality and embryonic outcomes (Dunning *et al*., 2010; Li *et al*., 2021; Song *et al*., 2018). Given that FAO generates significantly more ATP compared to glycolytic metabolism, oocytes accumulate lipid reserves as an efficient energy source for embryonic growth (Paczkowski *et al*., 2013). The yolk, a lipid-rich structure, serves as a vital nutrient and energy reservoir for developing embryos. Following fertilization, most yolk lipids are hydrolyzed into fatty acids or packaged into lipoproteins within the yolk syncytial layer (YSL) and transported to the body of the developing embryos (Quinlivan and Farber, 2017); this process is essential for facilitating critical cell signaling and proliferation event required for organogenesis (Lv *et al*., 2018). In this context, maternally expressed *cpt1* genes ensure adequate energy supply for fertilization and early cleavages.

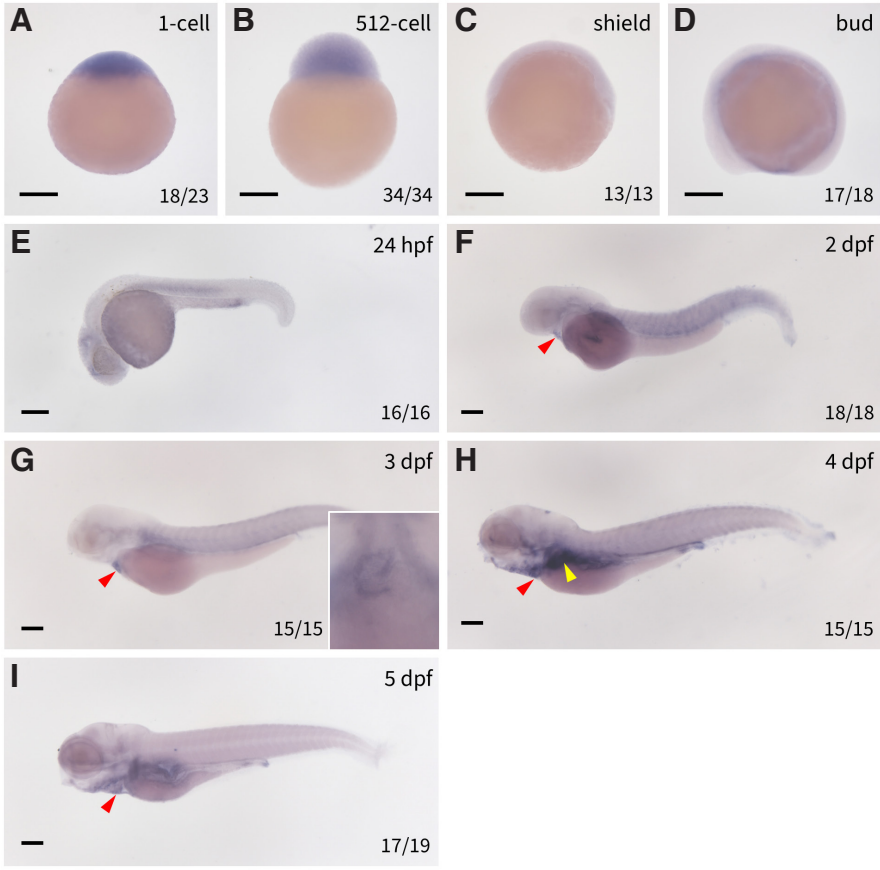
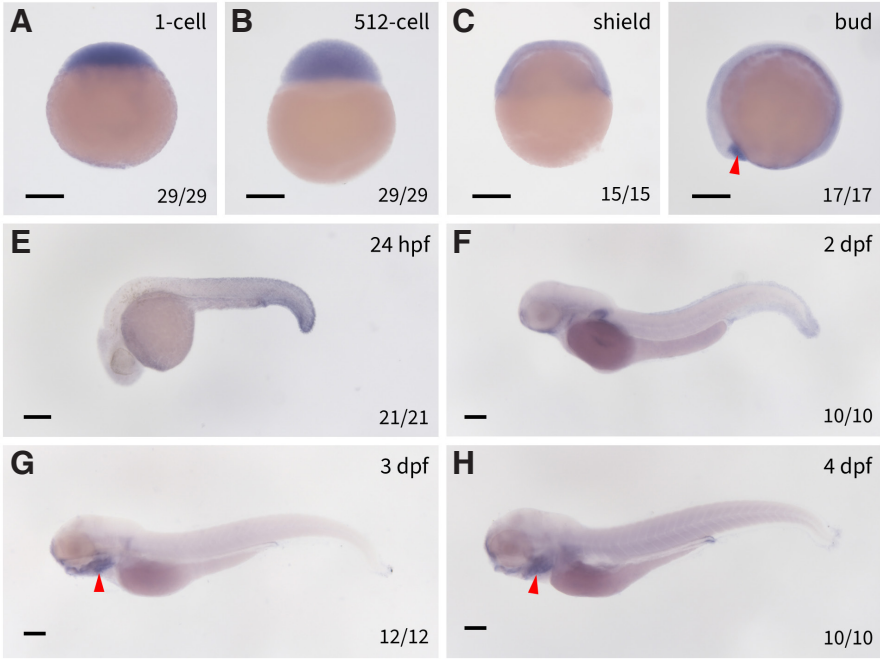


Fig. 4. Spatiotemporal expression of *cpt1b* in zebrafish embryos. Expression of *cpt1b* in **(A)** 1-cell stage embryos, (B) 512-cell embryos, (C) shield stage embryos, (D) bud stage embryos, (E) 24 hpf embryos, (F) 2 dpf embryos, (G) 3 dpf embryos (the inset is a ventral view of the embryo showing the expression of *cpt1b* in the heart), (H) 4 dpf embryos and (I) 5 dpf embryos. The red triangles denote the hearts, and the yellow triangle denotes the developing liver. Scale bars: 200 µm.



The expression profiles of CPT1 family genes exhibit tissue- and organ-specific variability, with each paralog displaying a distinct distribution pattern (Wang *et al*., 2021). In human and mice, *CPT1A* is primarily expressed in the liver, with additional expression in the brain and heart, whereas *CPT1B* is predominant in skeletal muscle, heart, and adipose tissues (Schlaepfer and Joshi, 2020; Esser *et al*., 1996). Although both *cpt1aa* and *cpt1ab* are co-orthologs of mammalian *Cpt1a* with 77% amino acid conservation, the present study demonstrates that *cpt1aa* is predominantly expressed in the liver and transiently in the brain during early embryogenesis (Fig. 1), whereas *cpt1ab* exhibits a broader expression profile similar to that of *cpt1b*, including expression in the heart, muscle, and intestines (Figs. 3, 4). The distinct expression patterns between *cpt1aa* and *cpt1ab* strongly suggest that they have undergone substantial divergent subfunctionalization and/or neofunctionalization following TGD event ~320 million years ago (Li *et al*., 2020), although definitive attribution of these mechanisms requires careful comparative analysis with non-TGD vertebrates (Braasch *et al*., 2016; Thompson *et al*., 2021). Conversely, despite sharing only 53% protein sequence identity between *cpt1ab* and *cpt1b*, their share convergent expression patterns particularly in cardiac and somitic tissues; however, it remains unclear whether this similarity arises from coincidence or convergent evolution driven by natural selection. Regardless, this expression pattern similarity implies potential genetic complementation between *cpt1ab* and *cpt1b*, which should be taken into account in subsequent experiments.

The heart is highly reliant on FAO for energy production, particularly postnatally, which correlates with high Cpt1 expression levels in this organ. In the developing lamb and rat hearts, both *Cpt1a* and *Cpt1b* are expressed, with their expression levels fluctuating dynamically throughout development and postnatal growth (Bartelds *et al*., 2004; Lavrentyev *et al*., 2004). In neonatal mouse hearts, *Cpt1b* expression is significantly upregulated, accompanied by

Fig. 5. Spatiotemporal expression of *cpt1a2b* in zebrafish embryos. Expression of *cpt1a2b* in **(A)** 1-cell stage embryos, **(B)** 512-cell embryos, **(C)** shield stage embryos, **(D)** bud stage embryos (the read triangle denotes the polster cells), **(E)** 24 hpf embryos, **(F)** 2 dpf embryos, **(G)** 3 dpf embryos (the read triangle denotes the pharyngeal endoderm) and **(H)** 4 dpf embryos. The read triangle denotes the pharyngeal endoderm. Scale bars: 200 µm.

a marked increase in FAO activity, which is essential for hypertrophic growth and maturation of cardiomyocytes (Cao *et al*., 2019). Previous studies have shown that Cpt1 inhibition prevents ventricular wall thinning and slows the progression of heart failure during the early stages of myocardial infarction (MI) in dogs (Lionetti *et al*., 2005). More recent investigations have revealed that both Cpt1a and Cpt1b can promote mouse cardiomyocyte proliferation and enhance cardiac function post-MI (Li *et al*., 2023; Tang *et al*., 2025). In zebrafish, FAO and *cpt1b* have also been demonstrated to be essential for cardiomyocyte proliferation and ventricular regeneration (Zhao *et al*., 2024; Cheng *et al*., 2024). Consistent with these findings, the present study shows that both *cpt1ab* and *cpt1b* are expressed in the developing zebrafish heart. Ventricular ablation suppresses cardiac *cpt1b* expression without affecting *cpt1ab* levels, and *cpt1b* expression gradually returns to normal level as the zebrafish heart regenerates. These results suggest that *Cpt1a* and *Cpt1b* may exert both overlapping and distinct functions during cardiac regeneration. Further comparative analyses of *Cpt1a* and *Cpt1b* single mutants, as well as double mutants, are therefore necessary to clarify their differential roles in this process.

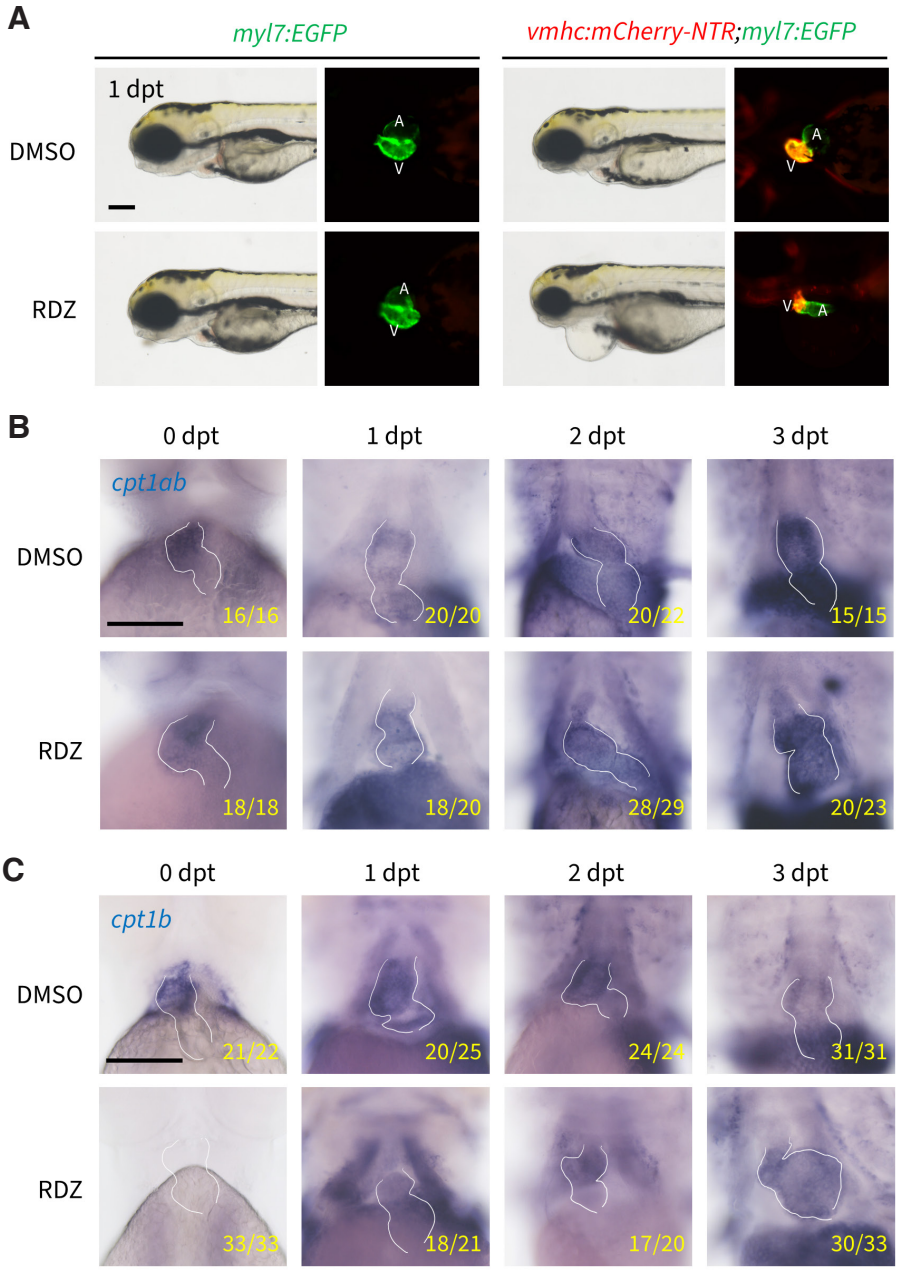


Fig. 6. Differential expression dynamics of *cpt1ab* and *cpt1b* during zebrafish heart regeneration. **(A)** Ronidazole (RDZ) treatment induces ventricular cardiomyocyte ablation. The embryos were imaged at 1 day post treatment (dpt). V, ventricle; A, atrium. **(B)** Whole-mount *in situ* hybridization shows temporal *cpt1ab* expression during zebrafish heart regeneration. **(C)** Temporal *cpt1b* expression during zebrafish heart regeneration. The white lines demarcate embryonic heart boundaries. Scale bars: 500 μm.

Materials and Methods

Zebrafish husbandry

All zebrafish used in this study were of the AB strain. Transgenic zebrafish lines used in this study were *Tg(vmhc:mCherry-NTR)* and *Tg(myl7:EGFP)*. Adult zebrafish were maintained in a dedicated zebrafish breeding facility (ESEN Science & Technology, Beijing, China) under a diurnal light cycle of 14 hours of light and 10 hours of darkness. The fish were fed with artemia twice daily. The water temperature was consistently maintained at approximately 28°C, with a pH level of around 7.2 and a conductivity of approximately 500 μS/cm. Zebrafish embryos were obtained through natural mating and were cultivated in an incubator at 28.5°C. Zebrafish embryo staging followed the standard developmental timelines established by Kimmel *et al.,* (1995) (Kimmel *et al*., 1995).

Phylogenetic analysis

Orthologous CPT1 sequences were identified through the Ensembl Comparative Genomics portal, leveraging orthology predictions from zebrafish *cpt1* gene pages. Curated protein sequences for all analyzed species (Supplementary Data 1) underwent multiple sequence alignment using ClustalX 2.1 with default parameters. Phylogenetic reconstruction was performed via maximum-likelihood methodology implemented in ClustalX, followed by visualization and annotation using the Interactive Tree of Life platform (iTOL v7; https://itol.embl.de) (Letunic and Bork, 2024).

RNA probe synthesis

Zebrafish *cpt1* cDNA fragments were amplified from an embryonic cDNA library using primers in Table 1. PCR products were cloned into a customized blunt-end cloning vector downstream of the T7 promoter. Positive

TABLE 1

PRIMERS USED TO CLONE PROBE TEMPLATE FOR CPT1 GENES

Gene	Zfin Gene ID	Forward primer	Reverse primer
cpt1aa	ZDB-GE-NE-060503-925	AAAACCCTGTGGAGTCTCTG	TTGTTTCCGAAGCGATGAGA
cpt1ab	ZDB-GE-NE-030131-3250	CACTGGACAGCTATGGCAAA	GATATCAAGCATCGCCTGTT
cpt1b	ZDB-GE-NE-041010-9	GAAGCACATGGATACGATCC	GTGTCAATGAGATTGAGCTG
cpt1a2b	ZDB-GE-NE-050417-155	GTCGTATTCCTGGTACGGTC	GCGTCATGGAAGCTTCATAT

clones were identified through colony PCR using T7 + gene-specific forward primers and validated by Sanger sequencing. Verified constructs were used to amplify templates for probe synthesis using T7 + forward primer pairs. Purified amplicons served as templates for *in vitro* transcription with T7 RNA polymerase (M0251S, New England Biolabs, Ipswich, USA) and DIG RNA Labeling Mix (11277073910, Sigma-Aldrich, St. Louis, USA). Riboprobes were purified by lithium chloride precipitation, resuspended in nuclease-free water, and stored at -80°C.

Whole mount *in situ* hybridization

In situ hybridization was performed as previously described (Thisse and Thisse, 2008). Zebrafish embryos at specified stages were dechorionated and fixed overnight with 4% paraformaldehyde (PFA, P6148, Sigma-Aldrich), followed by dehydration using a graded series of methanol and stored at -20°C until use. For *in situ* hybridization, embryos were first rehydrated, permeabilized with proteinase K, and re-fixed with 4% PFA. After pre-hybridization, embryos were incubated with heat-denatured DIG-labeled riboprobes overnight at 65°C in hybridization buffer. Subsequently, unbound probes were thoroughly removed via sequential washes and embryos were blocked with blocking reagent prior to incubation with anti-DIG antibody solution (1:3000; 11093274910, Sigma-Aldrich) at 4°C overnight. Unbound antibody was removed by serial washing and signals were developed with NBT/BCIP solution. Reactions were closely monitored and stopped by PBS washes when optimal signal-to-background ratio was achieved.

Ventricular ablation

Ronidazole (RDZ, S4062, Selleck, Houston, USA) was dissolved in DMSO to a final concentration of 2 M and the stock solution was aliquoted and stored at -20°C. To ablate ventricular cardiomyocytes, 36 hpf *Tg(vmhc:mCherry-NTR;cmlc2:EGFP)* embryos were incubated with egg water containing either DMSO or 0.4 mM RDZ in dark for 18 hours. After ablation, the embryos were rinsed twice with fresh egg water and transferred to fresh egg water for further culture.

Imaging

To image the embryos, samples were rinsed with PBS solution, cleared in glycerol until they sank to the bottom, and mounted in glycerol on a double concave slide. Images were acquired with a Zeiss Zoom V16 stereomicroscope (Oberkochen, Germany) equipped with a Zeiss Axiocam 208 camera and Zen 3.1 software.

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Author contributions

X.C. and Y.H. - conceptual and design of this study. X.C., W.H., C.K., Z.D., and H.C. - data collection and statistical analysis.Y.H. - manuscript preparation.

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