

# Duplicated zebrafish insulin-like growth factor binding protein-5 genes with split functional domains: evidence for evolutionarily conserved IGF binding, nuclear localization, and transactivation activity

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**ABSTRACT** Insulin-like growth factor binding protein (IGFBP)-5 is a secreted protein that binds to IGF and modulates IGF actions. IGFBP-5 is also found in the nucleus of mammalian cells and has transactivation activity. The structural basis of this transactivation activity and its role in mediating IGF-independent actions are not clear. Here we report that there are 2 *igfbp-5* genes in zebrafish and other teleost fish. In zebrafish, *igfbp-5a* and *-5b* are expressed in spatially restricted, mostly nonoverlapping domains during early development. The IGF binding site is conserved in both zebrafish IGFBP-5s, and they are both secreted and capable of IGF binding. Both proteins contain a consensus bipartite nuclear localization signal and were found in the nucleus when introduced into cultured cells. Although zebrafish IGFBP-5b possesses transactivation activity, zebrafish IGFBP-5a lacks this activity. Mutational analysis demonstrated that 2 unique amino acids in positions 22 and 56 of IGFBP-5a are responsible for its lack of transactivation activity. These findings suggest that the duplicated zebrafish IGFBP-5s have evolved divergent regulatory mechanisms and distinct biological properties by partitioning of ancestral structural domains and provide new evidence for a conserved role of the IGF binding, nuclear localization, and transactivation domain of this multifunctional IGFBP.—Dai, W., Kamei, H., Zhao, Y., Ding, J., Du, Z., Duan, C. Duplicated zebrafish insulin-like growth factor binding protein-5 genes with split functional domains: evidence for evolutionarily conserved IGF binding, nuclear localization, and transactivation activity. *FASEB J.* 24, 2020–2029 (2010). [www.fasebj.org](http://www.fasebj.org)

**Key Words:** IGFBP • IGF-independent action • gene expression • subfunctionalization

REGULATION OF PEPTIDE growth factor actions by secreted binding proteins has emerged as a common mechanism in cellular signaling. Among the most extensively studied examples are the insulin-like growth factor binding proteins (IGFBPs). Six distinct IGFBPs, designated as IGFBP-1 to -6, have been characterized in

humans and other mammals (1, 2). These IGFBPs bind to IGFs with equal or even greater affinities than do the IGF-1 receptors (IGF-1Rs) and modulate the distribution, stability, and biological activities of IGFs.

IGFBP-5 is the most conserved member of the IGFBP family. Mammalian IGFBP-5 has a highly conserved N domain where the primary ligand binding domain (LBD) is located (3) and a conserved C domain containing a nuclear localization signal (NLS) (1, 4). The central variable linker (L) domain contains several post-translational modification sites. Studies have suggested that IGFBP-5 is a multifunctional protein. In the blood, IGFBP-5 can form a ternary complex with IGF and the acid labile subunit (ALS). This ternary complex controls the efflux of IGFs from the vascular space and prolongs the half-lives of IGFs (1). In cultured cells, IGFBP-5 has been shown to inhibit IGF activities by binding to IGF and inhibiting IGF binding to the cell surface IGF-1 receptor (IGF-1R) (5). IGFBP-5 has also been shown to potentiate IGF actions *via* its interactions with extracellular matrix (ECM) components (6–8). Recent studies suggest that IGFBP-5 itself can act as a growth factor with cellular effects that are not dependent on its IGF-binding ability (9–12). In addition to these findings based on studies in various mammalian cell culture systems, recent mouse genetic studies have begun to shed light on the IGF-dependent and -independent actions *in vivo* (13–15).

Despite this progress, the molecular and biochemical mechanisms underlying the IGF-independent actions of IGFBP-5 are still poorly understood. Recent studies suggest that mammalian IGFBP-5 is not only secreted but also can be found in the nucleus and has the ability to interact with nuclear proteins (4, 16–20). Furthermore, the IGFBP-5 N domain has been shown to have a functional transactivation (TA) domain that is separable from its IGF binding site (19, 21). The *in vivo* roles of the nuclear IGFBP-5 and its TA domain in mediating the IGF-independent actions, however, are not clear.

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Addressing this issue using the mouse model is difficult because of the redundancy issues inherited with the mammalian systems and because of the multiple functionality nature of IGFBP-5.

Like in mammals, the IGF signaling systems in teleosts are composed of IGF ligands, receptors, and IGFBPs (22). Recent studies have suggested that many teleost fish, including zebrafish, experienced an additional genome wide duplication event (23, 24). For instance, there are 2 functional genes for IGF-1R, IGFBP-1, IGFBP-2, and IGFBP-6 in zebrafish (25–29). In this study we present evidence that zebrafish and several other fishes possess 2 functional *igfbp-5* genes. Exploiting the availability of 2 zebrafish IGFBP-5s, we show that the duplicated zebrafish IGFBP-5s have evolved distinct biological properties by partitioning of ancestral structural domains, such as the TA domain.

## MATERIALS AND METHODS

All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless stated otherwise. Restriction enzymes were purchased from Promega (Madison, WI, USA). *Taq*DNA polymerase and Vent DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotide primers and cell culture media were purchased from Invitrogen (Carlsbad, CA, USA).

### Animals

Wild-type zebrafish (*Danio rerio*) were maintained on a 14/10 h light-dark cycle at 28°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method (30). To inhibit pigmentation, embryo medium was supplemented with 0.003% (w/v) *N*-phenylthiourea. All experiments were carried out in accordance with the guidelines established by the University Committee on the Use and Care of Animals at the University of Michigan (<http://www.ucuca.umich.edu>).

### Molecular cloning and molecular evolutionary analyses

Two zebrafish cDNAs encoding IGFBP-5-like sequences were found by database search and by screening a cDNA library. Their full-length cDNA sequences were determined by 5' and 3' rapid amplification of cDNA ends (RACE) using the SMART RACE kit (Clontech, Mountain View, CA, USA). Amino acid sequences of IGFBP-5s were aligned by ClustalX (31). Phylogenetic analyses were conducted using full-length amino acid sequences by the minimum evolution method in MEGA4 (32). The GenBank accession numbers of various IGFBPs are listed in Supplemental Table 1. The structures of the 2 zebrafish *igfbp-5* genes were determined by comparing full-length cDNAs and the zebrafish genome sequence (<http://www.genome.ucsc.edu/cgi-bin/hgBlat>). Synteny analysis was carried out based on *Homo sapiens* Build 36.3, *Mus musculus* Build 37.1, *Danio rerio* Zv7, *Takifugu rubripes* FUGU 4.0, and *Gasterosteus aculeatus* BROAD S1. Genes used for this study are summarized in Supplemental Table 2.

### Reverse transcription (RT)-PCR and whole-mount *in situ* hybridization

Total RNA was isolated from embryos and adult zebrafish tissues using TRIzol reagent (Invitrogen). One microgram of total RNA

was reverse transcribed to single-strand cDNA using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed with 3 sets of primers (*igfbp-5a*: 5'-GGGTACATGTGGACGAGGA-3' and 5'-GAAAGAGCCATCACTCTGGAA-3'; *igfbp-5b*: 5'-GGGAGTGTGTACGAACGAGAA-3' and 5'-TCCTGTACAGTTAGGCAGTA-3';  $\beta$ -*actin*: 5'-GCCGGTTTTGCTGGAGATGAT-3' and 5'-ATGGCAGGGGTGTTGAAGGTC-3') using *Taq*DNA polymerase.

For whole-mount *in situ* hybridization analysis, plasmids containing complete CDS (*igfbp-5a*: 807 bp; *igfbp-5b*: 798 bp) or partial 3' UTRs (*igfbp-5a*: 501 bp; *igfbp-5b*: 485 bp after stop codon) were linearized by restriction enzyme digestion, followed by *in vitro* transcription reactions with either T7 or SP6 RNA polymerase (Promega), to generate antisense or sense riboprobes using DIG RNA labeling mix (Roche, Indianapolis, IN, USA). The specificity of the riboprobes was verified by dot-blot assay, and they did not cross-react with each other's target. Hybridization was carried out as described previously (25).

### Construction of plasmids

To produce purified recombinant proteins for biochemical assays, the open reading frames (ORFs) (with the stop codon deleted) of zebrafish IGFBP-5a and IGFBP-5b and human IGFBP-5 were amplified by PCR and subcloned into pcDNA3.1(-)/myc-His A expression vector (Invitrogen) at *Xho*I and *Hind*III sites. To determine the subcellular localization of the 2 zebrafish IGFBP-5s, their ORFs (with the stop codon deleted) were amplified by PCR and subcloned into pCS2<sup>+</sup>/enhanced green fluorescent protein (EGFP) expression vector as reported previously (33). The construction of human IGFBP-4:EGFP and IGFBP-5:EGFP constructs was already reported (19). To produce the GAL4 DNA-binding domain (DBD) and IGFBP-5a N-domain fusion protein, a DNA fragment corresponding to the N domain of zebrafish IGFBP-5a was generated by PCR and subcloned into pBIND vector (Promega). The pBIND constructs containing human IGFBP-5 or zebrafish IGFBP-5b N domain were already reported (19, 21). The IGFBP-5 N-domain mutants were generated by PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) as described previously (21). Primers used for constructing these plasmids are listed in Supplemental Table 3. All constructs were sequenced at the University of Michigan DNA Sequencing Core Facility.

### Expression and purification of recombinant proteins

Myc- and 6xHistidine-tagged human and zebrafish IGFBPs were produced and purified following previously reported procedures (27). The purified proteins were quantified using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The purity was confirmed by silver staining and Western immunoblot using an anti-c-myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Their IGF binding abilities were determined by ligand blot using digoxigenin-labeled human IGF-1 following published procedure (34).

### Cell-growth assay

The biological activities of various IGFBP-5s were studied using the MTS assay (Promega) in cultured human embryonic kidney (HEK) 293 cells and human osteosarcoma (U2OS) cells. Cells were cultured in DMEM (HEK 293) or McCoy's 5A (U2OS) supplemented with 10% FBS, penicillin, and streptomycin in a humidified-air atmosphere incubator containing 5% CO<sub>2</sub>. After 20 h serum starvation, 25 nM purified IGFBP was added in the presence or absence of 25 nM IGF-1 (Novozymes GroPep,

Adelaide, SA, Australia). The assays were terminated after 48 h, following the manufacturer's instructions.

### Subcellular localization of zebrafish IGFBP-5a and -5b

U2OS cells were transiently transfected with an IGFBP:EGFP expression vector using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Twenty-four hours after transfection, cells were washed with 1× PBS, fixed by 4% paraformaldehyde for 1 h, and stained with 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The cells were washed, mounted, and examined under a fluorescence microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan). Images were acquired using Leica TCS SP5 confocal microscope with the Leica LAS AF software (Leica Microsystems, Wetzlar, Germany).

### Transcription activation assay

Mammalian one-hybrid transcription activation assay was performed as described previously (21). The TA activities of various IGFBPs were also examined in a zebrafish embryonic cell line (ZF4). ZF4 cells were cultured in DMEM/F12 supplemented with 10% FBS, penicillin, and streptomycin in a humidified-air atmosphere incubator containing 5% CO<sub>2</sub> at 28°C. FuGENE 6 (Roche) was used for transfection in ZF4 cells. Twenty-four hours after transfection, cells were washed and lysed. TA activity was quantified using the Dual-Luciferase Reporter Assay System (Promega). To detect the expression level of GAL4 (DBD):IGFBP N-domain fusion proteins, equal amounts of cell lysates were separated by 12.5% SDS-PAGE and transferred to Immobilon P membranes (Millipore, Billerica, MA, USA), followed by Western immunoblot using an anti-GAL4 (DBD) (RK5C1) antibody (Santa Cruz Biotechnology) and an anti-tubulin antibody (Sigma-Aldrich, St. Louis, MO, USA).

### Statistics

All values are represented as means ± SD. Statistical differences among experimental groups were analyzed by 1-way analysis of variance (ANOVA), followed by the Newman-Keuls multiple comparison test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Identification of 2 *igfbp-5* genes in zebrafish and other teleost fish species

By searching public databases, screening a cDNA library, and performing 5'- and 3'-RACE experiments, we identified and cloned 2 distinct zebrafish genes (GenBank accession numbers GQ892882 and AY100478). For reasons evident thereafter, we termed them as *igfbp-5a* and *-5b* and the encoded proteins as IGFBP-5a and -5b. As shown in Fig. 1A, zebrafish IGFBP-5a has a putative signal peptide of 19 amino acids (aa) and a mature protein of 249 aa. IGFBP-5b has a putative signal peptide of 17 aa and a mature protein of 248 aa. Comparison of the 2 zebrafish IGFBP-5 sequences with 6 human IGFBPs revealed that they share the highest sequence identities with that of human IGFBP-5 (47–52%; see Supplemental Table 4). Their sequence identities to human IGFBP-3 are 36 and

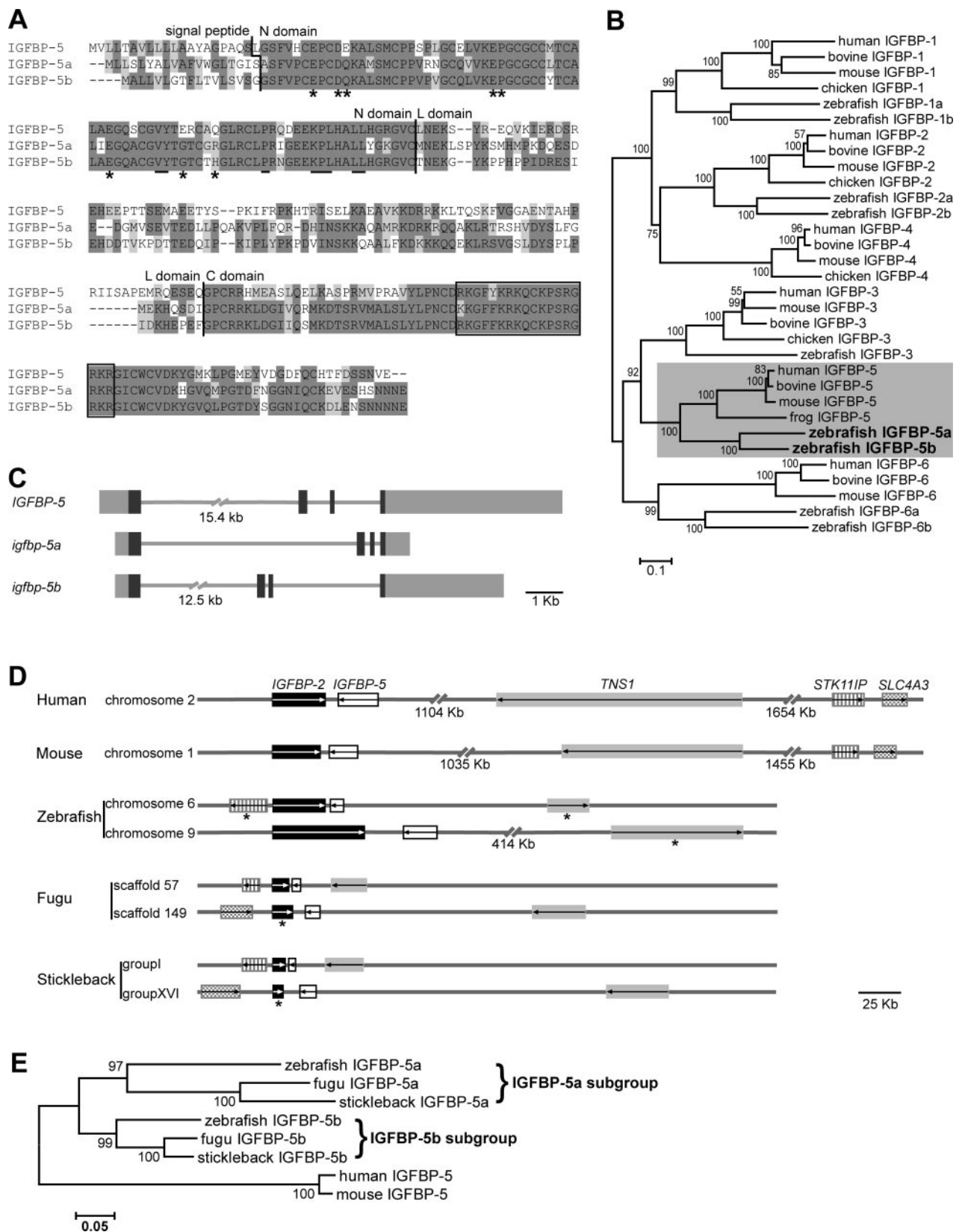
37%, and below 30% to other human IGFBPs. There is a typical IGFBP motif in the N domain and a thyroglobulin type-1 repeat in the C domain in both zebrafish IGFBP-5s. Both proteins contain a consensus LBD motif (3) in the N domain and an NLS motif (4) in the C domain (Fig. 1A).

Phylogenetic analysis grouped both proteins into the IGFBP-5 subgroup (Fig. 1B). The 2 zebrafish *igfbp-5* genes also share similar exon/intron organization with the human *IGFBP-5* gene: they all contain 4 exons and 3 introns (Fig. 1C). Although zebrafish *igfbp-5a* is located on LG6, *igfbp-5b* is on LG9. The 2 zebrafish *igfbp-5* genes are adjacent to the 2 previously reported *igfbp-2* genes (28), arranged in a tail-to-tail fashion (Fig. 1D). This is very similar to the situations in the human and mouse genomes (35).

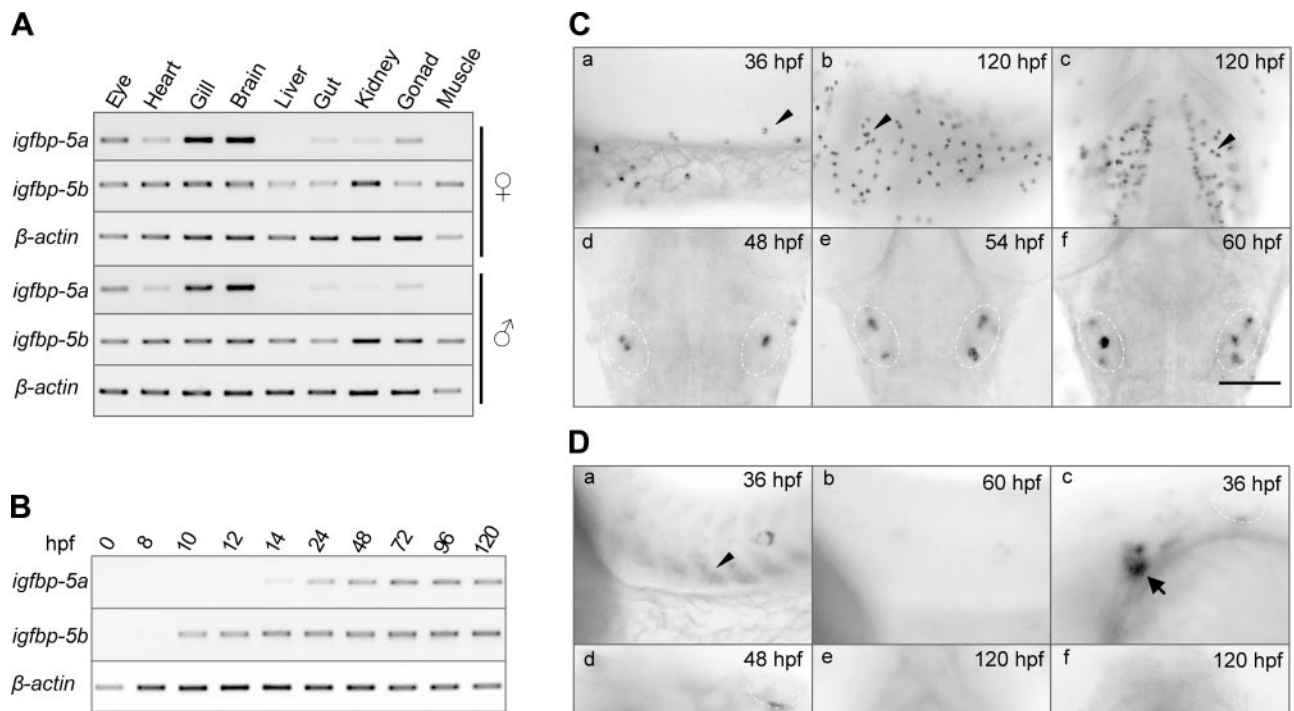
To determine whether there are 2 *igfbp-5* genes in other teleost fish, we searched the fugu, stickleback, medaka, and tetraodon genome databases and found that they all contain 2 *igfbp-5* genes. Again, the 2 *igfbp-5* genes are adjacent to 2 *igfbp-2* genes in a tail-to-tail fashion in all these teleost genomes (Fig. 1D). Likewise, there are several other syntenic genes (*TNS1*, *STK11IP*, *SLC4A3*) (Fig. 1D). Phylogenetic analysis indicated that the duplication of the *igfbp-5a/b* subfamily likely originated from a genome duplication event that occurred early during ray-fin fish evolution (Fig. 1E).

### The duplicated *igfbp-5* genes exhibit distinct expression patterns

As shown in Fig. 2A, in adult tissues, *igfbp-5a* mRNA was detected in brain and gill at high levels. It was also detected in eye, heart, gut, kidney, and gonad, but not in liver and muscle by RT-PCR (Fig. 2A). In comparison, *igfbp-5b* mRNA was expressed in all adult tissues examined. There were no obvious gender differences (Fig. 2A). During early development, *igfbp-5a* mRNA was not detectable until 14 h postfertilization (hpf). It gradually increased from 14 to 72 hpf and was maintained at high levels thereafter (Fig. 2B). In 8-hpf embryos, *igfbp-5b* mRNA was detected at low levels. Starting from 12 hpf, *igfbp-5b* mRNA levels maintained at high levels thereafter (Fig. 2B). The results of whole-mount *in situ* hybridization analysis are shown in Fig. 2C, D. We first detected *igfbp-5a* mRNA at 20 hpf in a small number of cells on the surface of the yolk sac and yolk tube, and this became more evident at 36 hpf (Fig. 2Ca). The number of *igfbp-5a* mRNA-expressing cells increased as the embryos grew (Fig. 2Cb). At 96 and 120 hpf, *igfbp-5a* mRNA was also highly expressed in cells spreading in the gill filament regions (Fig. 2Cc). We also detected *igfbp-5a* mRNA in a small number of cells located within the inner ear (Fig. 2Cd–f). In contrast, *igfbp-5b* mRNA was primarily detected in differentiating somites, gill arches, pectoral fin, and some neural tissues (Fig. 2D). Its expression in the somites disappeared at 60 hpf (Fig. 2Db). At 36 and 48 hpf, *igfbp-5b* mRNA was also highly expressed in the epithelial cells in the otic vesicles (Fig. 2Dc, d). In larvae (120 hpf), *igfbp-5b* mRNA was detected in the layers of cells surrounding the gill cartilage (Fig. 2De) and some cells in the brain (Fig.



**Figure 1.** There are 2 *igfbp-5* genes in zebrafish and other teleost fish. *A*) Sequence alignment of human IGFBP-5 and zebrafish IGFBP-5a and -5b. Identical and similar amino acid residues are darkly and lightly shaded, respectively. Vertical lines indicate the boundaries between signal peptide, N, L, and C domains. Residues in the N domain known to be critical for IGF binding (LBD) are underlined. NLS is shown in box. Asterisks indicate residues that are critical for the TA activity of human IGFBP-5. *B*) Phylogenetic tree of the IGFBP family. Values on branches are percentages of replicate trees in which the genes clustered together in the bootstrap test (1000 replicates). Branch lengths are drawn in units of 0.1 aa substitutions/site. *C*) Structure of human *IGFBP-5* and zebrafish *igfbp-5a* and *-5b*. Exons are shown as boxes and introns as lines. Lightly and darkly shaded areas represent (continued on next page)



**Figure 2.** Zebrafish *igfbp-5a* and *-5b* exhibit distinct spatial and temporal expression patterns. **A)** RT-PCR analysis of *igfbp-5a* and *-5b* mRNAs in female and male adult zebrafish tissues. **B)** RT-PCR analysis of *igfbp-5a* and *-5b* mRNAs in zebrafish embryos at the indicated stages. Developmental stages are shown at top, hpf, hours postfertilization. **C)** *In situ* hybridization analysis of *igfbp-5a* mRNA in whole-mount zebrafish embryos. Developmental stage is at top right in each panel. *a, b*) Lateral view of yolk tube/sac region with anterior at left. *c*) Ventral view of gill arch region with anterior at top. Arrowheads indicate one representative epidermal cell. *d–f*) Dorsal view of head region with anterior at top. Dotted line indicates position of otic vesicles. **D)** *In situ* hybridization analysis of *igfbp-5b* mRNA. *a, b*) Lateral view of trunk region with anterior at left. Arrowhead indicates signals in somites. *c, d*) Lateral view of head region with anterior at left. Dotted line indicates position of otic vesicles. *e*) Ventral view of gill arch region with anterior at top. Arrows indicate signals in gill arch. *f*) Dorsal view of head region with anterior at top. Arrowhead indicates signal in midbrain. Scale bars = 100  $\mu$ m.

2Df). These results suggest that the 2 *igfb-5* genes are expressed in nonoverlapping domains during development.

### Both IGFBP-5a and -5b are secreted proteins, and they both bind to IGF and modulate IGF actions

To test whether zebrafish *igfbp-5a* and *-5b* encode functional IGFBPs, recombinant zebrafish IGFBP-5a and -5b and human IGFBP-5 were produced in HEK 293 cells and purified from the culture medium. HEK 293 cells were used because of their high transfection efficiency. Human and zebrafish IGFBP-5s had apparent sizes of  $\sim$ 36 kDa on SDS-PAGE (Fig. 3A), and they were all able to bind IGF-1, as shown by ligand blot (Fig. 3A).

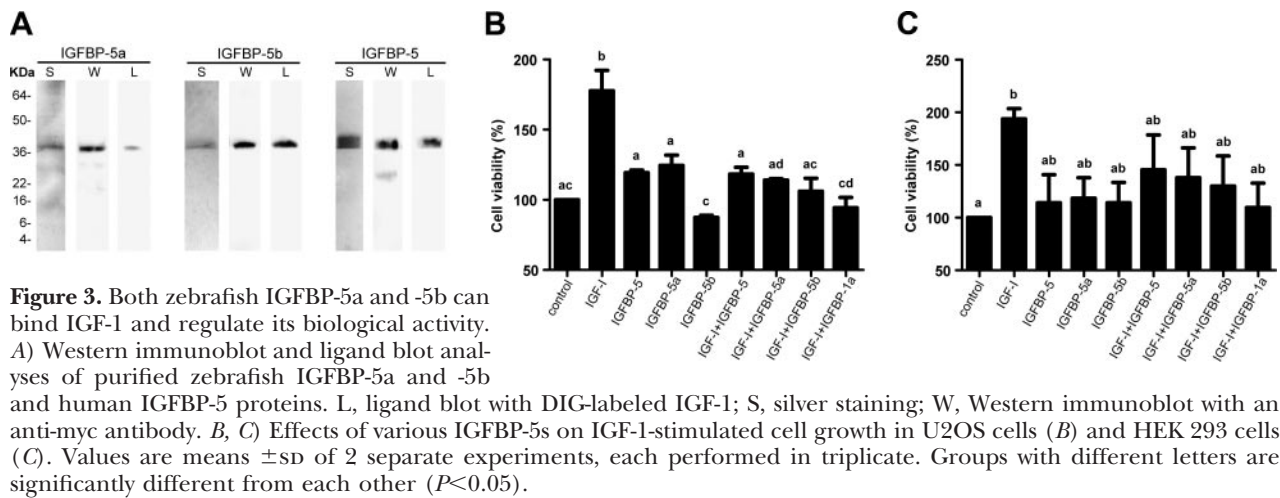
We next determined the biological activities of zebrafish IGFBP-5a and -5b and compared them to that of

human IGFBP-5. As shown in Fig. 3B, addition of IGF-1 to cultured U2OS cells caused a significant increase in cell growth. When human IGFBP-5, zebrafish IGFBP-5a, or zebrafish IGFBP-5b was added together with IGF-1 at a 1:1 M ratio, they abolished the IGF-1-induced increase. Addition of any one of these IGFBP-5s alone had little effect. Similar results were also obtained in HEK 293 cells (Fig. 3C). These results suggest that zebrafish *igfbp-5a* and *-5b* encode secreted proteins that bind IGF-1 and modulate IGF-1 actions.

### Both IGFBP-5a and -5b are localized in the nucleus, but only IGFBP-5b has TA activity

Previous studies have shown that human IGFBP-5 is not only secreted but can also be found in the nucleus of

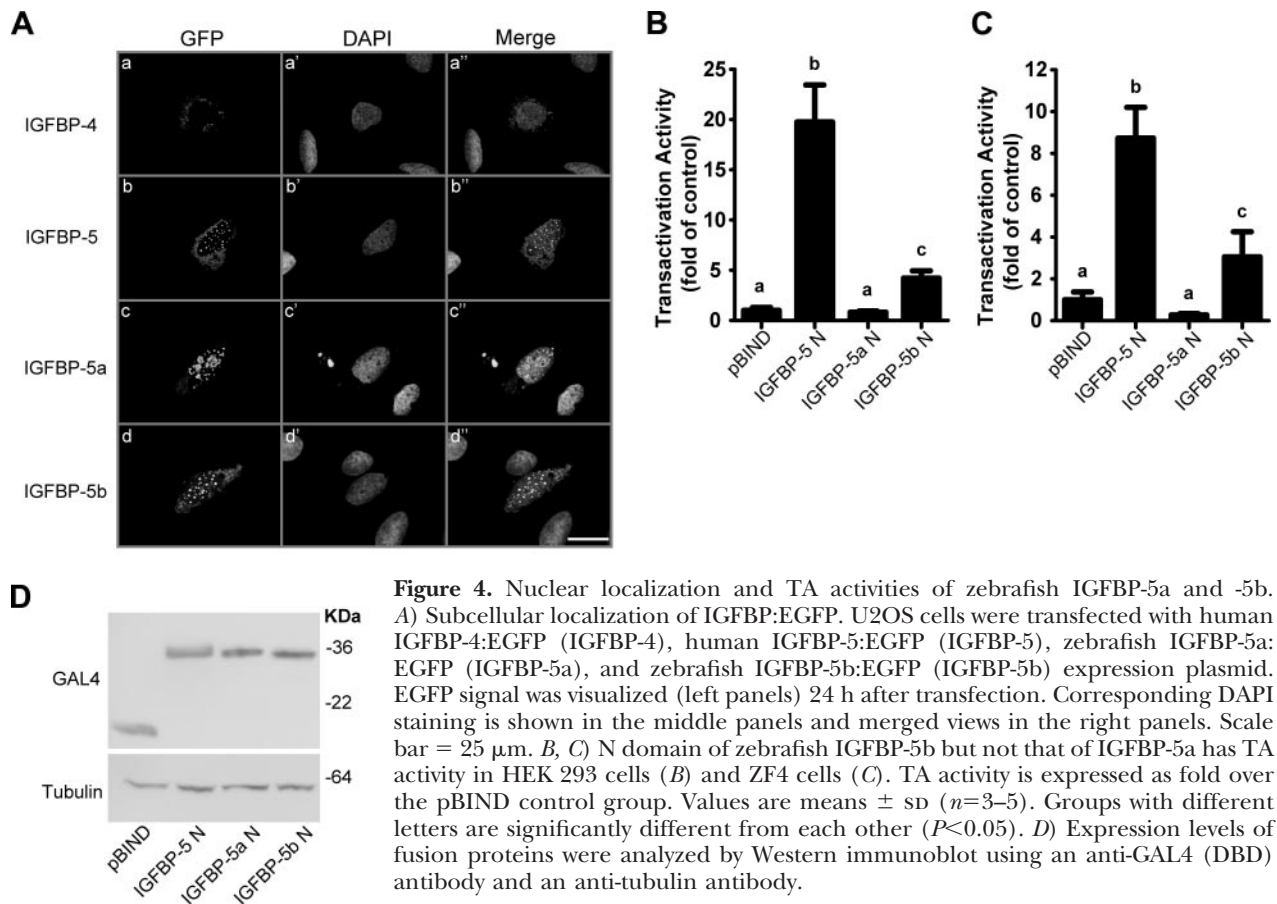
UTRs and ORF, respectively. **D)** Chromosomal loci of *IGFBP-5* genes in various vertebrate genomes. Arrows indicate the transcript orientation. *IGFBP-2*, *IGFBP-5*, *TNS1*, *STK11IP*, and *SLC4A3* are shown as black box, open box, gray box, vertical-line filled box, and wave-line filled box, respectively. Asterisk indicates partial transcript information from gene annotation. (Chromosomal locus of *SLC4A3* in zebrafish is unknown.) **E)** Phylogenetic analysis of several teleost IGFBP-5a and -5b. Values on branches are percentages of replicate trees in which genes clustered together in the bootstrap test (1000 replicates). Branch lengths are drawn in units of 0.05 aa substitutions/site.



cultured mammalian cells and mouse embryos (4, 17–19, 21). Furthermore, the IGFBP-5 N domain has TA activity (19, 21). Because both zebrafish IGFBP-5s contain a consensus NLS in their C domains (Fig. 1A, open box), we investigated the possible nuclear localization of zebrafish IGFBP-5a and -5b. U2OS cells were chosen for subcellular localization analysis because of their large and flattened cell bodies. When cells were transfected with human IGFBP-4:EGFP, the EGFP signal was detected only in the cytoplasm (Fig. 4A). But when cells were transfected with human IGFBP-5:EGFP, the EGFP signal was seen in the nucleus. Like human

IGFBP-5:EGFP, zebrafish IGFBP-5a:EGFP, and -5b:EGFP were also found in the nucleus (Fig. 4A). Similar results were also observed in HEK 293 cells (data not shown).

We next investigated whether zebrafish IGFBP-5a and/or -5b have any TA activity. HEK 293 cells were used because of the high transfection efficiency (21). The zebrafish IGFBP-5b N domain caused a GAL4-dependent TA 4-fold greater than the pBIND control group when tested in HEK 293 cells (Fig. 4B). In contrast, the zebrafish IGFBP-5a N domain did not cause any significant increase. As reported previously (19, 21), the human IGFBP-5 N

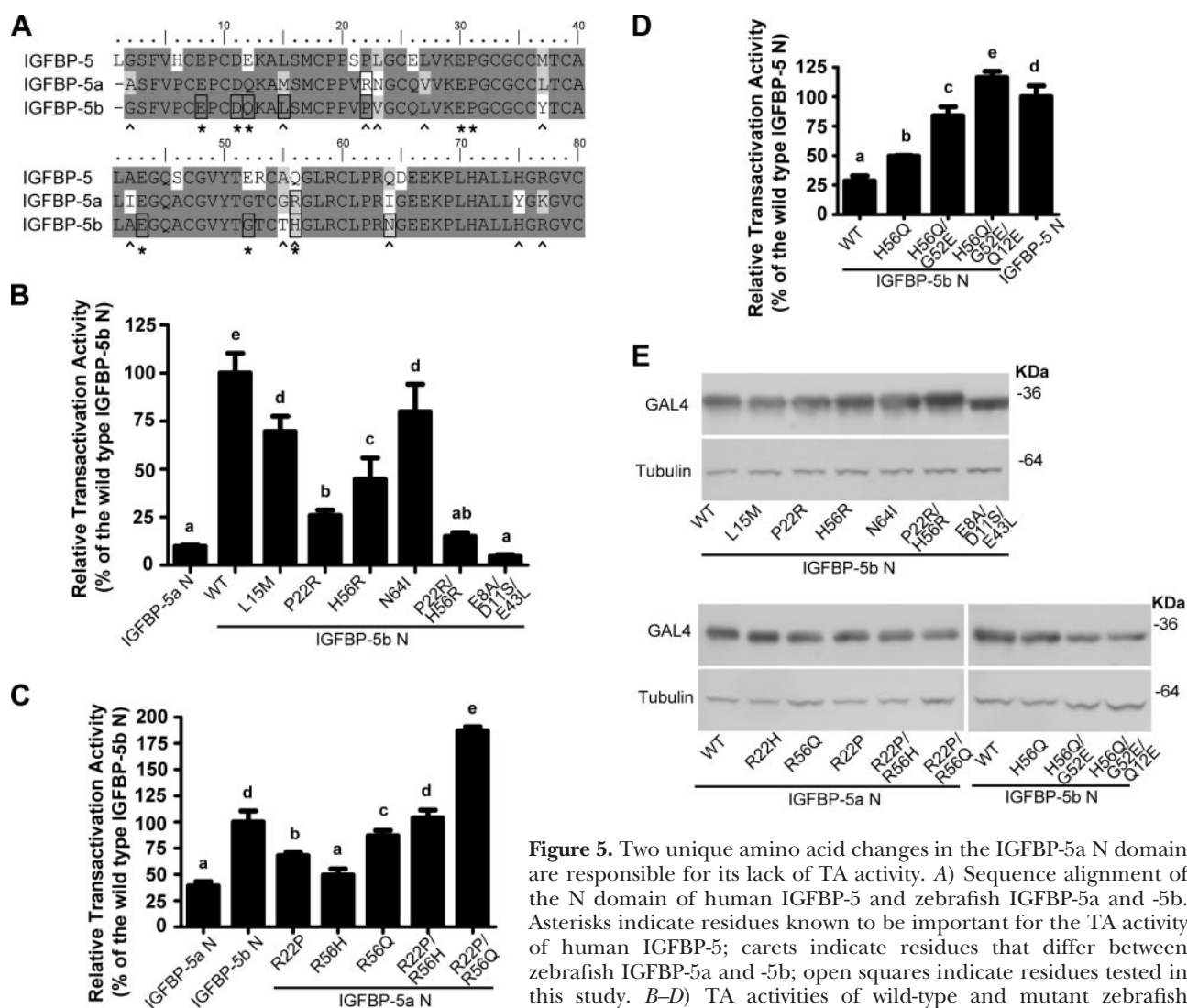


domain caused a 20-fold increase in activating the reporter gene expression. To rule out the possibility of species-specific effect, the TA activities of these fusion proteins were also tested in ZF4 cells, a cell line derived from zebrafish embryos. In these zebrafish cells, human IGFBP-5 still had the strongest activity (9-fold increase over the pBIND control,  $P < 0.001$ ) and the zebrafish IGFBP-5b N domain had significant activity (3-fold increase,  $P < 0.05$ ). Again, the zebrafish IGFBP-5a N domain had no activity (Fig. 4C). Western immunoblot analysis revealed that expression levels of these fusion proteins were similar (Fig. 4D), thus excluding the possibility that the difference was due to different levels of protein expression and/or degradation.

### Several unique residues in the IGFBP-5 N domain are critical for the TA activity

The 2 zebrafish IGFBP-5s share the same domain arrangement, high sequence identity, nuclear localiza-

tion, and the ability to bind IGFs, but only IGFBP-5b has TA activity. Taking advantage of this finding, we compared the amino acid sequences of these 2 highly homologous proteins with that of human IGFBP-5. Among the 8 residues in the human IGFBP-5 N domain that are known to be critical for its TA ability (21), 5 are conserved in zebrafish IGFBP-5a and -5b (Fig. 5A, asterisk). When 3 of these conserved residues in zebrafish IGFBP-5b were substituted with their corresponding residues from human IGFBP-1 (E8A/D11S/E43L), the TA activity was abolished (Fig. 5B). Because zebrafish IGFBP-5b, but not IGFBP-5a, has TA activity, we focused on the 12 residues that differed between these 2 zebrafish IGFBP-5s in this region (Fig. 5A, caret). Among the 4 tested, we found that changing P22 or H56 in IGFBP-5b to the corresponding residue in IGFBP-5a reduced the TA activity by  $>50\%$ , and the P22R/H56R double mutant had essentially no TA activity (Fig. 5B). In comparison, the L15M and N64I



**Figure 5.** Two unique amino acid changes in the IGFBP-5a N domain are responsible for its lack of TA activity. *A*) Sequence alignment of the N domain of human IGFBP-5 and zebrafish IGFBP-5a and -5b. Asterisks indicate residues known to be important for the TA activity of human IGFBP-5; carets indicate residues that differ between zebrafish IGFBP-5a and -5b; open squares indicate residues tested in this study. *B–D*) TA activities of wild-type and mutant zebrafish IGFBP-5b and -5a. Indicated constructs were introduced into HEK 293 cells together with a GAL4 reporter plasmid by transient transfection. Values are expressed as percentages of the wild-type zebrafish IGFBP-5b N-domain group (*B*, *C*) or the human IGFBP-5 N-domain group (*D*). Values are means  $\pm$  sd ( $n=3$ ). Groups with the same letters are not significantly different from each other ( $P < 0.05$ ). *E*) Expression levels of mutant fusion proteins were analyzed by Western immunoblot using an anti-GAL4 (DBD) antibody and an anti-tubulin antibody.

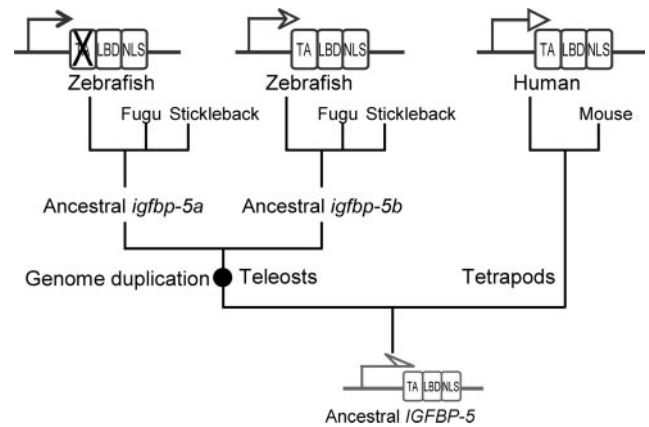
mutants had only moderate effects. These results suggest that R22 and R56 in the IGFBP-5a N domain are largely responsible for its lack of TA activity. To further test whether these 2 positions are sufficient to establish the TA activity, we changed R22 and R56 in the zebrafish IGFBP-5a N domain to the corresponding residues of IGFBP-5b in individual and double mutants. The R22P mutant had significant TA activity. The R22P/R56H double mutant had TA activity comparable to that of zebrafish IGFBP-5b (Fig. 5C). Taken together, our results suggest that the 2 different amino acids in positions 22 and 56 are responsible for the different TA activity observed in the 2 zebrafish IGFBP-5s.

We also investigated the structural determinants accounting for the different activities observed between human IGFBP-5 and zebrafish IGFBP-5b. Among the 8 residues in the human IGFBP-5 N domain that are known to be critical for its TA ability (21), 3 of them differ between zebrafish IGFBP-5b and human IGFBP-5 (Fig. 5A, asterisk). Changing the zebrafish IGFBP-5 residue at position 56 into the corresponding residue from the human sequence resulted in a significant increase in its activity (R56Q and R22P/R56Q mutants in Fig. 5C and H56Q mutant in Fig. 5D). We further generated double and triple zebrafish IGFBP-5b mutants, H56Q/G52E and H56Q/G52E/Q12E, by changing residues from the zebrafish IGFBP-5b into the corresponding ones from the human IGFBP-5 sequence. The H56Q/G52E mutant had 80% activity compared to the human IGFBP-5 (Fig. 5D), and the H56Q/G52E/Q12E mutant fully achieved the same high TA activity as human IGFBP-5. All mutants were expressed at comparable levels as shown by immunoblot (Fig. 5E). These results indicate that the difference in TA activities observed between human IGFBP-5 and zebrafish IGFBP-5b is due to their different amino acid residues in positions 12, 52, and 56.

## DISCUSSION

In this study we identified 2 *igfbp-5* genes in zebrafish. Several lines of evidence indicated that they are co-orthologs of human *IGFBP-5*: 1) sequence comparison at the protein level showed that they share the highest identity with human IGFBP-5, 2) phylogenetic analysis grouped the 2 zebrafish IGFBPs in the IGFBP-5 cluster, 3) the gene structure and exon/intron size are most similar to human *IGFBP-5*, and 4) the chromosome loci indicated conserved synteny with human *IGFBP-5*. We also found 2 *igfbp-5* genes in other teleost fish. Both the conserved synteny and phylogenetic analyses indicate that the duplication event that produced these 2 genes happened early in teleost evolution (Fig. 6).

In humans and mice, *IGFBP-5* mRNA has been detected in a wide range of tissues and cell types. Analyzing the expression patterns in zebrafish, we found that the 2 duplicate *igfbp-5* genes diverge in expression pattern both spatially and temporally. In adult tissues, although *igfbp-5b* is expressed in all the tissues examined at relatively high levels, *igfbp-5a* is most strongly expressed in brain and gill.



**Figure 6.** Proposed model for the gene expression and functional divergence of *igfbp-5* genes in teleost fish. The ancestral *IGFBP-5* gene was duplicated early in teleost evolution as the result of a genome duplication event. The duplicated *igfbp-5* genes may have undergone subfunctionalization at the levels of expression pattern, through divergent expression regulation (indicated by different arrows), and protein functionality, through the loss of critical structural motifs, such as the TA domain in the case of zebrafish *igfbp-5a*.

During embryogenesis, *igfbp-5b* is expressed earlier than *igfbp-5a*. More interestingly, they are expressed in distinct tissues and cells. Although *igfbp-5a* expression is restricted in the epidermal cells and in the inner ear, *igfbp-5b* is expressed in somites, branchial arches, pectoral fin, and several domains in the brain. The divergent temporal and spatial expression patterns indicate that the cis-regulatory elements in these 2 genes may have diverged after the duplication event. Studies of duplicated genes in zebrafish indicated that this may be a common mechanism for diversification (36, 37). Further studies are needed to determine the conserved and divergent cis-regulatory elements in these 2 zebrafish *igfbp-5* genes.

Mammalian IGFBP-5 is a multifunctional protein that contains several structural modules/domains. We performed molecular, biochemical and cell biological approaches to identify structural components that have diversified in the duplicate zebrafish IGFBP-5s. We found that although some of the domains are preserved in both genes (LBD and NLS), others (TA) diverged during evolution (Fig. 6). Protein function usually requires intra- and intermolecular domain interactions. Therefore, the related domains usually coevolve (38). It has been shown that TA activity and nuclear localization are well correlated in the 6 IGFBPs, with IGFBP-3 and -5 showing nuclear localization and possessing the highest TA activity (21). It is intriguing to ask why IGFBP-5a preserves the NLS while losing the TA domain. One possible explanation from the structural point of view is that the conserved stretch of basic residues in the C domain has multiple functional roles. In addition to being a functional NLS (4, 19), this region is also involved in IGFBP-5 interaction with ALS, heparin, and ECM components (39, 40). Therefore, selection force may conserve these residues even if one of their functions become unneces-



sary. The LBD motif and the TA domain have been shown to be structurally separable and functionally independent (19). It is possible that IGFBP-5a may have lost the TA activity while retaining its IGF-binding function.

The 2 zebrafish IGFBP-5s share the same domain arrangement, high sequence identity, and the ability to bind IGFs, but only zebrafish IGFBP-5b has TA activity. Taking advantage of this finding, we were able to identify the key residues critical for the TA activity. By swapping their different residues, we discovered that 2 residues at positions 22 and 56 are both necessary and sufficient for the TA activity in zebrafish IGFBP-5s. These results indicate that IGFBP-5a lacks TA activity because of its unique residues in these critical positions. This conclusion is also consistent with our current understanding of gene evolution. After gene duplication, it is thought that the duplicated genes are likely to be retained if they acquire nonredundant functions (41). The high divergence of the TA domains observed in the duplicated zebrafish *igfbp-5* paralogs may account for an adaptation or specialization of function of these 2 genes. We speculate that this may not be unique to zebrafish, as aligning the IGFBP-5 N domain sequences of zebrafish, fugu, and stickleback together with that of human and mouse suggests that position 56 showed clear divergence in the 2 branches. Specifically, it is an R in all members of the IGFBP-5a group, whereas Q or H in the IGFBP-5b group (Supplemental Fig. 1). The other position, 22, identified in this study does not seem to be conserved in the 2 groups, suggesting that this residue change is a more recent event during evolution in zebrafish. When we tested the TA activity in ancestral IGFBPs in amphioxus (unpublished results), we found that they do exhibit high TA activity like the human IGFBP-5, suggesting that this activity has an ancient origin.

It has been shown that zebrafish contain 2 *igf-1* genes, 2 *igf-2* genes, and 2 *igf-1r* genes (26, 42). In addition, zebrafish have 2 *igfbp-1* genes, 2 *igfbp-2* genes, and 2 *igfbp-6* genes (27–29). In this study, we provide evidence that there are 2 functional *igfbp-5* genes in zebrafish and other teleost fish, *igfbp-5a* and *igfbp-5b*. We show that the duplicated *igfbp-5*s exhibited nonoverlapping expression patterns during zebrafish embryogenesis. We also determined the structural changes accounting for functional divergence by mapping critical amino acid changes in the TA domain. These findings provide insight into the evolution of the IGFBP gene family and lay the foundations for further elucidation of the physiological functions of the nuclear IGFBP-5 and its TA activity *in vivo*. FJ

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