Two Zebrafish *hsd3b* Genes Are Distinct in Function, Expression, and Evolution

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HSD3B catalyzes the synthesis of δ4 steroids such as progesterone in the adrenals and gonads. Individuals lacking HSD3B2 activity experience congenital adrenal hyperplasia with imbalanced steroid synthesis. To develop a zebrafish model of HSD3B deficiency, we characterized 2 zebrafish *hsd3b* genes. Our phylogenetic and conserved synteny analyses showed that the tandemly duplicated human *HSD3B1* and *HSD3B2* genes are coorthologs of zebrafish *hsd3b1* on chromosome 9 (Dre9), whereas the gene called *hsd3b2* resides on Dre20 in an ancestral chromosome segment, from which its ortholog was lost in the tetrapod lineage. Zebrafish *hsd3b1*(Dre 9) was expressed in adult gonads and headkidney, which contains interrenal glands, the zebrafish counterpart of the tetrapod adrenal. Knockdown of *hsd3b1*(Dre 9) caused the interrenal and anterior pituitary to expand and pigmentation to increase, resembling human *HSD3B2* deficiency. The zebrafish *hsd3b2*(Dre 20) gene was expressed in zebrafish early embryos as maternal transcripts that disappeared 1 day after fertilization. Morpholino inactivation of *hsd3b2*(Dre 20) led to embryo elongation, which was rescued by the injection of *hsd3b2* mRNA. Thus, zebrafish *hsd3b2*(Dre 20) evolved independently of *hsd3b1*(Dre 9) with a morphogenetic function during early embryogenesis. Zebrafish *hsd3b1*(Dre 9), on the contrary, functions like mammalian *HSD3B2*, whose deficiency leads to congenital adrenal hyperplasia. **(*Endocrinology* 156: 2854–2862, 2015)**

HSD3B genes encode 3β-hydroxysteroid dehydrogenase/Δ(5)-Δ(4)-isomerase, which catalyzes the formation of δ(4)-3-ketosteroids from δ(5)-3β-hydroxysteroids, a necessary step in the biosynthesis of mineralocorticoids, glucocorticoids, and sex hormones (1). When circulating glucocorticoid level is low or in stressed condition, the hypothalamus of the brain stimulates the pituitary to secrete ACTH, a peptide derived from the proteolytic cleavage of its precursor proopiomelanocortin (POMC) (2). ACTH targets the adrenal to stimulate the secretion of glucocorticoids that cope with demand. When present at a high level, glucocorticoids inhibit the hypothalamus and the pituitary from secreting ACTH, thus maintaining homeostasis of the hypothalamic-pituitary-adrenal axis.

Humans have 2 *HSD3B* genes, *HSD3B1* and *HSD3B2*, which are arranged in tandem and transcribed in the same direction. All known human HSD3B deficiencies have mutations in *HSD3B2*. HSD3B2 deficiency is an autosomal recessive disorder with decreased steroid production leading to excessive ACTH secretion and congenital adrenal hyperplasia (3). HSD3B2-deficient males have underdeveloped external reproductive organs, whereas females exhibit either normal sexual differentiation or mild virilization (4).

Zebrafish has become a useful animal model for developmental, behavioral, and physiological studies. The adrenal counterpart in zebrafish and other teleosts is located inside the kidney (5) and therefore is called the interrenal gland. Steroidogenic genes including *hsd3b* genes in the

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Abbreviations: DHEA, dehydroepiandrosterone; DIG, digoxigenin; dpf, days postfertilization; eef1a1a, eukaryotic translation elongation factor 1 α1a; hpf, hours postfertilization; HPI, hypothalamic-pituitary-interrenal; Hsa, Homo sapiens; HSD3B, 3beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4)-isomerase; HYB,hybridization buffer; MO, morpholino; P5, pregnenolone; PBST, 0.1% Tween 20/PBS; PFA, paraformaldehyde; POMC, proopiomelanocortin; SSC, Saline Sodium Citrate.

interrenal glands are regulated by transcription factor encoded by *ff1b* (6). The stress response system is also conserved in zebrafish, and termed the hypothalamic-pituitary-interrenal (HPI) axis. The negative feedback loop of the HPI axis in zebrafish is already active at 2 days postfertilization (dpf), at which time pituitary corticotrophs already show decreased *pomc* transcript levels after dexamethasone treatment (7). However, steroidogenic gene expression in the interrenal gland is sensitive to dexamethasone inhibition only starting at 3 dpf (7).

The human genome has a cluster of 2 functional and 5 *HSD3B* pseudogenes on human chromosome 6 (Homo sapiens [Hsa] 6), but the rat and mouse genomes have 5 and 6 functional *Hsd3b* genes, respectively (1). In the fish, a single *hsd3b* gene, which is expressed in the gonad, has been detected in medaka and trout genomes (8). Other teleost fish genomes, such as those of Japanese eel and zebrafish, contain multiple *hsd3b* genes. It is unclear whether *hsd3b* gene duplications occurred independently in different species in fish.

In zebrafish, 2 *hsd3b* genes have been described (9). To examine *hsd3b* expression and functions further, here we analyzed the 2 zebrafish *hsd3b* genes in more detail. We found that these 2 genes can be classified as an embryonic and a larval/adult form. We further showed that the adult form was the human ortholog and its deficiency led to a disturbance of the HPI axis. The embryonic form, on the contrary, had a role in embryo morphogenesis.

Materials and Methods

Maintenance of zebrafish (*Danio rerio*)

Fish were bred and maintained according to the procedures described in *The Zebrafish Book* (10). The AB and TL strains of zebrafish were kept in 28.5°C in egg water (60-μg/mL ocean salt) under the condition of a 14-hour light, 10-hour dark cycle daily. After fertilization, eggs were collected and cultured in 10-mL dishes at 28.5°C until 4 dpf. They were then put into 8-L tanks and fed with paramecia at least 4 times daily. Fish were kept in an automatic maintenance system after 2 or 3 weeks of age and fed with brine shrimp twice daily. This study was approved by Academia Sinica Institutional Animal Care and Utilization Committee, protocol number 12–03-339.

cDNA cloning and RT-PCR

Total RNA was extracted from zebrafish tissues using TRIzol reagent (Sigma). The 5′-end of *hsd3b1*(Dre9) cDNA was first amplified from ovarian RNA with a 5′-GTAGCTGAATTTA-AGGCGAGAGGAG-3′ primer designed according to a partial gene sequence from the database (BC074074.1). The sequence from this 5′-clone was used for the design of new primers (forward, 5′-AGCTTCTGAGGACTTGTTACACCTC-3′ and reverse, 5′-GCAATAATTGCTGAAGCTTTTATCC-3′) for the amplification of full-length *hsd3b1*(Dre9) cDNA from head kidney cDNA. The *hsd3b2*(Dre20) cDNA was also cloned from head kidney cDNA by PCR amplification using forward primer 5′-CGCAGTGTCGCACATCGTCTCAGG-3′ and reverse primer 5′-ACATGCCCATATATGCTTTTCGATGG-3′, the sequences of which were obtained from the sequence of a cDNA that we cloned before (11). Both *hsd3b1* and *hsd3b2* cDNAs were subcloned into pcDNA3 vector for expression in COS-1 cells.

Real-time PCR was performed in a Roche LightCycler 1.5 using QuantiFast SYBR green (QIAGEN). The eukaryotic translation elongation factor 1 α1a (eef1a1a) gene was used as an internal control for quantitative real-time PCR of all mRNAs. The sequences of real-time PCR primers used for analysis are listed as follows: *pomca* (forward), 5′-GCGCAGGAGGATCT-GAAGT-3′ and *pomca* (reverse), 5′-GGGAGGCTGTAGATG-GCTTT-3′; *hsd3b1* (forward), 5′-CTGGTTCGACTGTTGCT-GAA-3′ and *hsd3b1*(reverse), 5′-AAACAAGCGCTGCTCC-TTTA-3′; *hsd3b2* (forward), 5′-AGTGTCGCACATCGTCT-CAG-3′ and *hsd3b2* (reverse), 5′-CACCACACACACCTCTC-CTG-3′; and *eef1a1a* (forward), 5′-AGCAGCAGCTGAG-GAGTGAT-3′ and *eef1a1a* (reversed), 5′-TGGTTCTCT-TGTCGATTCCA-3′.

For RT-PCR analysis of *hsd3b1*(Dre9) mRNA, the synthetic oligonucleotides were forward: 5′-AGCTTCTGAGGACTT-GTTACACCTC-3′ and reverse: 5′-GTAGCTGAATTTAA-GGCGAGAGGAG-3′; and for *hsd3b2*(Dre 20) they were forward, 5′-GCATCGACTCTCACAGCAGAG-3′ and reverse, 5′-ACATGCCCATATATGCTTTTCGAT-3′ to amplify 579 and 588-bp fragments. To provide a RNA loading control, β-actin was amplified using the next primers: 5′-TCACACCTTC-TACAACGAGCTGCG-3′ and 5′-GAAGCTGTAGCCTCT-CTCGGTCAG-3′. After 25 cycles of PCR, PCR products were analyzed in agarose gel.

Cell culture and transient transfection

COS-1 cells were maintained in DMEM-10% FBS in 5% CO_2, 37°C. Transfections were performed with Lipofectamine 2000 along with 2.5-μg pcDNA3 vectors with or without *hsd3b* cDNAs when cells in 6-well plates were 60%–70% full. Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde (PFA)/PBS at room temperature for 10 minutes and washed twice with PBS before chromogenic reactions.

Chromogenic histochemical staining for 3β-hydroxysteroid dehydrogenase/Δ(5)-Δ(4)-isomerase

Histochemical staining for Hsd3b was performed using a protocol described previously. Embryos or COS-1 cells were fixed for 1 hour or 10 minutes, respectively, at room temperature in 4% PFA in PBS and washed twice with 0.1% Tween 20/PBS (PBST). The chromogenic reaction was incubated at room temperature with Hsd3b substrate (0.1-mg/mL etiocholan-3β-ol-17-one, 100-μg/mL dehydroepiandrosterone [DHEA] or 100-μg/mL pregnenolone [P5]) in 1.5-mg/mL β-nicotinamide adenine dinucleotide, 1-mg/mL BSA, 1% N,N dimethylformamide, 8.75mM EDTA for embryo staining only, and 1-mg/mL nitro-blue tetrazolium chloride. Reactions were monitored until sufficient signal intensities were obtained (8–20 h) and were terminated by washing in PBST followed by fixation in 4% PFA/PBS for 1 hour or 10 minutes, respectively. In control experiments, the substrate was replaced by the solvent. Color signals

were examined using a Leica Z16 APO microscope and photographs were taken using an AxioCam HRc camera (Zeiss).

For the quantitation of Hsd3b activities, chromogenic signals produced from cells were integrated using the MetaMorph Microscopy Automation and Image Analysis Software. The color signals from 5 random fields were converted into binary and then divided by areas (μm^2) to obtain specific Hsd3b activities.

RNA probe synthesis

Digoxigenin (DIG)-11-uridine triphosphate labeled RNA probe was prepared from *Nco*I-linearized *pGT-ff1b* using bacteriophage SP6 RNA polymerase for antisense probe (12). An antisense *pomc* riboprobe was transcribed with bacteriophage T7 RNA polymerase from the full-length cDNA contained in the *pCRII-TOPO* cloning vector and digested with *Xho*I (13). About 5-μg plasmid containing insert fragments were used as template to synthesize antisense RNA labeled with DIG RNA Labeling Mix (Roche). After incubation at 37°C for 2 hours, each 20-μL labeling reaction was terminated with 1-μL 0.5M EDTA. For each reaction, 5-μL 3M NaOAc (pH 5.2) and 50-μL isopropanol were added. After freezing at −20°C for 30 minutes, samples were centrifuged at 13 000 rpm, 4°C for 25 minutes. The pellet was rinsed by 70% ethanol and air dried. DIG-labeled RNA probes were dissolved in 50 μL of diethyl pyrocarbonate-treated H_2O, then quantified as described in manufacturer's guide and stored at −20°C.

Whole-mount in situ hybridization

Samples were collected and then fixed with 4% PFA in PBS (pH 7.4) overnight at 4°C, the chorion was then removed and embryos were dehydrated in methanol and stored at −20°C until use. Rehydration procedures were performed by the next steps: 75%, 50%, and 25% methanol/PBS for 10 minutes and then washed twice with PBST before digestion with proteinase K (10-μg/mL PBST) and was postfixed in 4% PFA for 30 minutes, again washed twice with PBST and hybridization buffer $(HYB)^-$ (50% formamide, 5× Saline Sodium Citrate (SSC), and 0.1% Tween 20) before prehybridization in HYB^+ (HYB^-, 50-mg/mL heparin, and 5-mg/mL torula RNA) for 2–5 hours at 68°C. DIG-labeled RNA probes were then added and allowed to incubate at 68°C overnight. Samples were then washed with HYB^- at 68°C for 5 minutes once, followed by several steps: 75%, 50%, and 25% HYB^-/2× SSC for 10 minutes, and once 2× SSC, twice 0.2× SSC for 1 hour at 68°C. They were washed with preblocking solution (150mM maleic acid, 100mM NaCl, and 0.1% Tween 20; pH7.5) for 5 minutes at room temperature. They were then incubated in blocking solution (5% lamb serum, 2-mg/mL BSA, and 0.1% Tween 20 in PBS) for 2–4 hours at room temperature. An aliquot of a 1:4000 dilution of anti-DIG-alkaline phosphate fragment antigen-binding fragments (Boehringer Mannheim) was added and incubation continued at 4°C overnight. After this incubation, samples were washed 3 times with preblocking solution (150mM maleic acid, 100mM NaCl, and 0.1% Tween 20; pH7.5) for 25 minutes and 3 times with PBST for 15 minutes and then equilibrated in detection solution (0.1M Tris [pH 9.5], 0.05M $MgCl_2$, and 0.1M NaCl) for 30 minutes. BM purple substrate (Roche) was used for detection. Reaction was stopped by 4% PFA when the desired signal appeared.

Phylogenetic and synteny analysis

Published amino acid sequences of Hsd3b proteins in 25 vertebrates were collected and analyzed by the phylogenetic program of Molecular Evolutionary Genetics Analysis software version 5.1 (15). The default values for parameters of this program were used for the construction of the phylogenetic tree using the neighbor-joining method (16). Conserved synteny analyses used the Synteny Database (17).

Morpholino (MO)

The MO sequences are as follows: *hsd3b1*MO1, GGAAATATCCACTAACCATCAAGAG; *hsd3b2*MO2, CTGCGTTGAAAGCCCGTCGAGTC; and control MO (*cyp17* sense), CAGTTGAATATAGCTGACAATGGCT.

Microinjection

MO was diluted into working concentrations of 4 μg/μL in Danieau solution (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, and 5mM HEPES; pH 7.6) before microinjection. The injection needles were selected with the opening of 5–10 μm in diameter at the end. Fertilized eggs before the first cleavage were collected and soaked in egg water (tap water containing ocean salt 60 μg/mL) at room temperature. To perform cytoplasmic microinjection, the egg was oriented on the egg holder to present the animal pole of the cytoplasm under a stereo microscope, then transferred to a plastic dish containing egg water and incubated at 28.5°C.

Cortisol measurement

About 70 embryos (10 hours postfertilization [hpf]) or 40 larvae (5 dpf) were used for each cortisol measurement. These samples were first frozen in liquid nitrogen, homogenized, and extracted with ethyl acetate. The organic layer was collected and vaporized. The pellet was dissolved in ELISA assay buffer and cortisol was measured using a Cortisol EIA kit (Cayman Chemical Co). Student's *t* test was used for statistical analysis.

Results

Phylogenetic and synteny analyses of zebrafish *hsd3b* genes

We previously cloned and analyzed a zebrafish *hsd3b* cDNAs (11). Here, we cloned a second *hsd3b* cDNA. To examine the evolutionary relationships of these 2 zebrafish *hsd3b* genes and those from other species, we built a phylogenetic tree using amino acid alignments of 25 selected HSD3B sequences (Figure 1). The 2 zebrafish *hsd3b* genes grouped as sisters in the tree, more closely related to each other than to Hsd3b protein from other species. Zebrafish Hsd3b proteins also grouped with the orthologous HSD3B1 and HSD3B2 proteins of humans rather than zebrafish Hsd3b7. Zebrafish *hsd3b* genes did not group with any single isotype of mammalian *Hsd3b* genes, some of which evolved by duplication of an ancestral gene in lineage-specific duplication events.

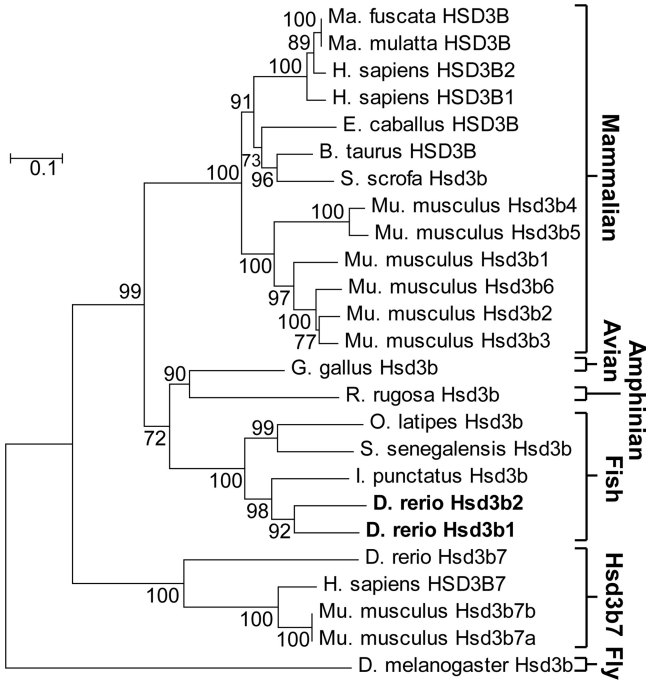

Figure 1. Phylogenic analysis of vertebrate *Hsd3b* genes. A phylogenetic tree was generated by the neighbor-joining method. Numbers indicate the bootstrap support for the branching pattern from 1000 replicates. The scale of 0.1 is the length that corresponds to a 10% sequence difference. *Bos taurus*, cattle; *Danio rerio*, zebrafish; *Drosophila melanogaster*, fly; *Equus caballus*, horse; *Gallus gallus*, chicken; *Homo sapiens*, human; *Ictalurus punctatus*, channel catfish; *Macaca fuscata*, Japanese macaque; *Macaca mulatta*, rhesus macaque; *Mus musculus*, mouse; *Oryzias latipes*, medaka; *Rana rugosa*, frog; *Sus scrofa*, wild pig; *Spilopelia senegalensis*, flatfish.

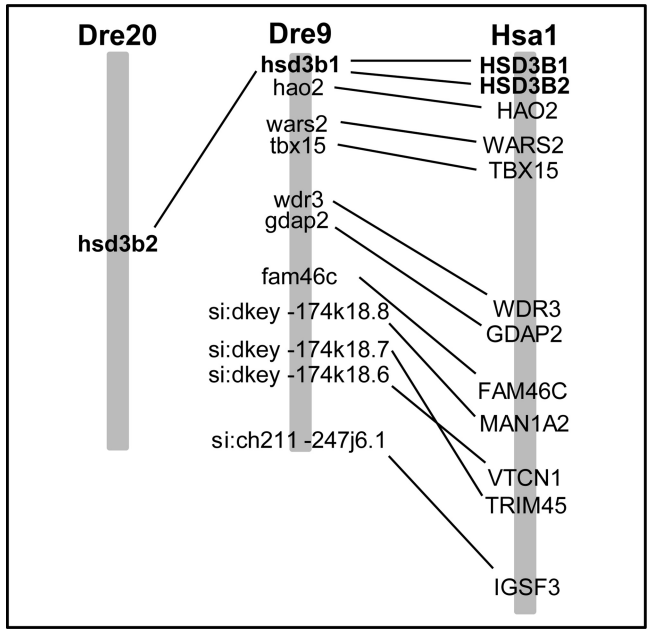

Figure 2. Conserved syntenies around *hsd3b* genes. Gene loci in conserved syntenies are listed in the order they appear on the zebrafish chromosome 9 (Dre9). Zebrafish chromosome 20 (Dre20) does not share significant conserved syntenic gene regions with Dre 9 or human chromosome 1 (Hsa1).

To investigate the evolutionary relationship of the zebrafish *hsd3b* genes, we examined their gene loci in the genome. The *hsd3b1* gene is located on zebrafish chromosome 9 (Dre9) in a region orthologous to the region on human chromosome 1 (Hsa1) that contains the tandemly duplicated *HSD3B1* and *HSD3B2* genes (Figure 2). This finding indicates that human *HSD3B1* and *HSD3B2* genes are co-orthologous to zebrafish *hsd3b1*(Dre9). The zebrafish *hsd3b2* gene (NP_997962, ENSDARG00000019747) is located in chromosome 20: 1,251,700–1,255,349 (Dre 20); this region is unrelated to the human chromosome segment containing *HSD3B* genes. Because it is not in a paralogous chromosome segment with Dre9 nor does it share conserved syntenies with the human *HSD3B1/HSD3B2* region, zebrafish *hsd3b2*(Dre 20) gene appears to duplicate independently from the rest of the genes.

Expression and enzymatic activities of 2 *hsd3b* genes

To understand whether one or both of the *hsd3b* genes encode active enzymes, we expressed zebrafish Hsd3b1 and Hsd3b2 by transfecting their expression plasmids into COS-1 cells for enzymatic activity assays. Using a chromogenic assay (14), we found that cells that expressed zebrafish Hsd3b1 or Hsd3b2 were stained dark blue after incubation with DHEA or P5, but no staining was observed after incubation with the solvent ethanol (Figure 3A). COS-1 cells transfected with an empty vector also did not stain. Quantitation of the staining data showed that both Hsd3b1 and Hsd3b2 were enzymatically active (Figure 3, B and C).

To investigate the expression patterns, we detected 2 zebrafish *hsd3b* mRNAs in various adult organs by quantitative RT-PCR. Both *hsd3b1* and *hsd3b2* mRNAs were abundantly expressed in the head kidney and gonads (Figure 4A). Their expression in other tissues was low or undetected.

In addition to expression in different adult tissues, we also examined the temporal expression of both *hsd3b* genes. Transcripts from *hsd3b2*(Dre20) were expressed from the 1-cell stage; its level peaked at bud stage and then down-regulated soon after 1 dpf (Figure 4B). The *hsd3b1*(Dre9) mRNA was detected after 1 dpf and continued past 4 dpf (Figure 4B). Thus, these 2 *hsd3b* genes differ in their temporal expression; *hsd3b2*(Dre20) was embryonically expressed, whereas *hsd3b1*(Dre9) was expressed later in the larvae and adults.

Knockdown of *hsd3b* gene expression by MO antisense oligonucleotides

To understand the roles of *hsd3b* genes, we designed antisense MO oligonucleotides (MOs) targeted against

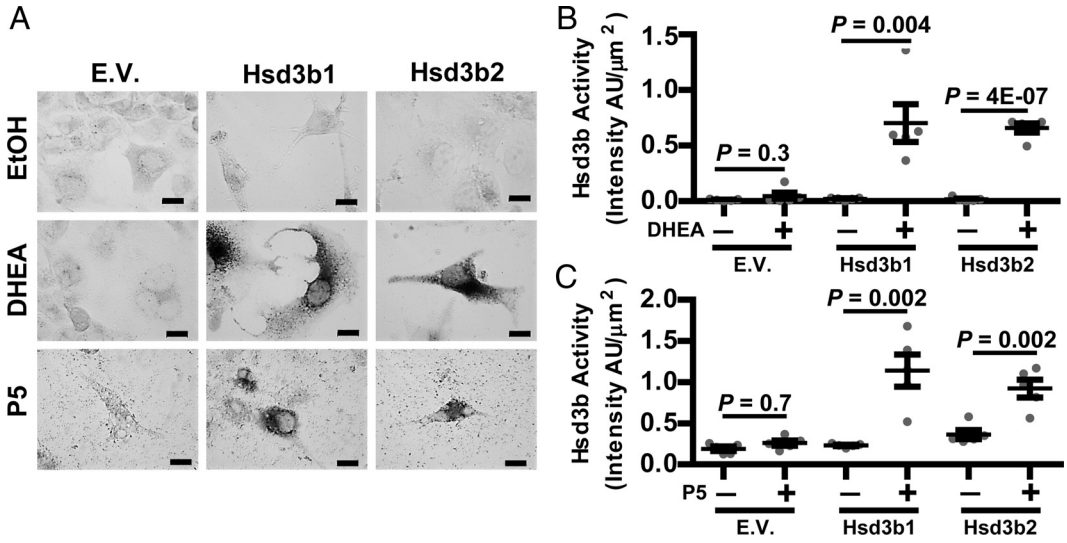

Figure 3. Zebrafish Hsd3b1 and Hsd3b2 expressed in COS-1 cells are enzymatically active. A, Chromogenic assays of HSD3B activities after COS-1 cells were transfected with expression plasmids harboring empty vector (E.V.), zebrafish *hsd3b1*, or *hsd3b2* cDNA. Cells were incubated with DHEA, P5, or ethanol for overnight staining using nitro-blue tetrazolium chloride. Scale bars, 50 μm. B and C, Quantitation of Hsd3b enzymatic activities. The staining intensities were quantified using the MetaMorph Microscopy Automation and Image Analysis Software attached to the microscope.

the donor site of intron 1 for *hsd3b1*(Dre9) or against the translational start site for *hsd3b2*(Dre20) (Figure 5A) and used an unrelated MO (a sense strand for *cyp17*, which we previously showed had no effect) as the negative control.

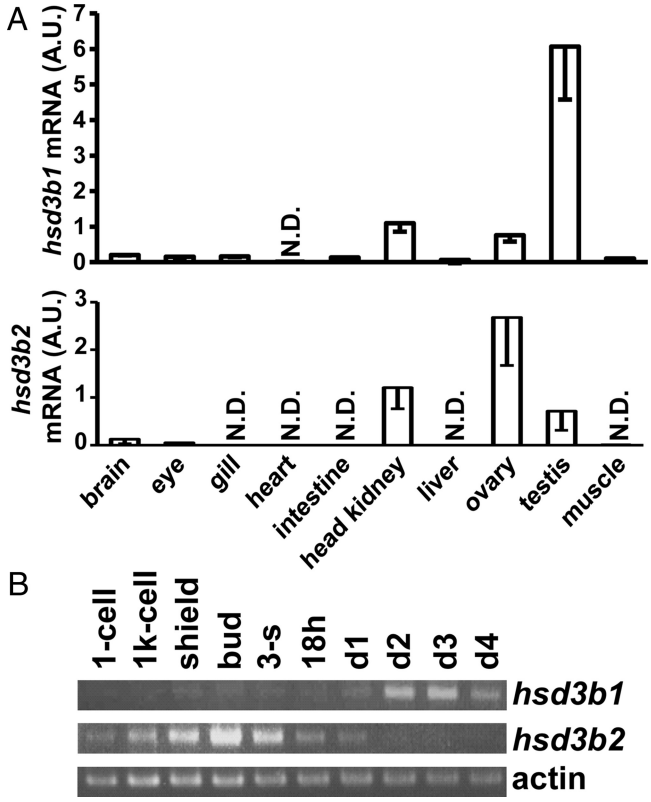

Figure 4. Expression of zebrafish *hsd3b1* and *hsd3b2* mRNAs. A, Quantitative RT-PCR analysis of *hsd3b1* and *hsd3b2* expression in different adult tissues. N.D., not detected. B, Gel showing RT-PCR analysis of *hsd3b1* and *hsd3b2* transcripts at different stages during early development. Actin is used as an internal control. 1k-cell, embryos with 1000 cells; 3-s, 3-somite stage; d1–d4, day 1 to day 4 after fertilization.

We tested whether the MOs were effective at the protein level by detecting Hsd3b enzymatic activities using a chromogenic assay. The enzymatic activity appeared in the interrenal primordia of embryos at 33 hpf when injected with control MO (Figure 5B), and this enzymatic activity was blocked by *hsd3b2*MO2. This indicates that Hsd3b2(Dre 20) worked in the interrenal primordia at 33 hpf. At 48 hpf, the enzymatic activity was blocked by *hsd3b1*MO1 at 48 hpf, indicating that the Hsd3b activity present at 48 hpf was encoded by *hsd3b1*(Dre 9).

To differentiate the activities of these 2 Hsd3b proteins further, we chose 50 hpf, when *hsd3b2*(Dre 20) mRNA was completely shut off. Strong Hsd3b enzymatic activity was detected in control MO-injected embryos (Figure 5C). This activity was reduced after the injection of *hsd3b1*MO1 but not by *hsd3b2*MO2 (Figure 5C). This result confirmed our finding that *hsd3b2* was not expressed in the 2-dpf embryo and therefore did not contribute to Hsd3b activity at 50 hpf. The marker of interrenal primordial cells (17), *ff1b*, was expressed after the injection of either *hsd3b1* or *hsd3b2* MOs, indicating that interrenal primordial cells were still present in all these conditions. This result indicates that the predominant interrenal Hsd3b at 50 hpf was encoded by *hsd3b1*(Dre 9).

hsd3b1(Dre 9) exerts a conserved role in the HPI axis

To understand the role of *hsd3b1*, we blocked its expression in zebrafish embryos by MO and examined their phenotypes. Embryos injected with control MO

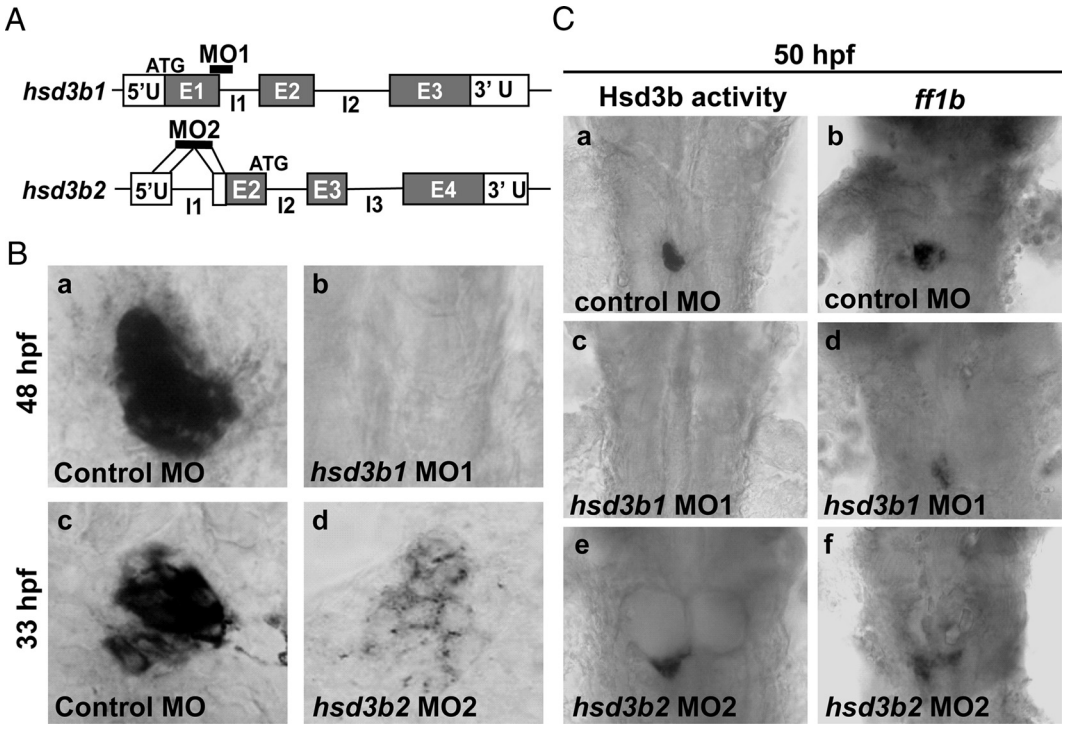

Figure 5. Efficient inhibition of Hsd3b activities by MOs. A, Locations and sequences of *hsd3b1 MO1* and *hsd3b2 MO2*. Exons (E) are shown as boxes, and coding sequences (CDS) are shaded. Introns (I) and flanking sequences are shown as horizontal lines. U, untranslated region. B, Hsd3b enzymatic activities detected by the chromogenic assay were abolished by *hsd3b1MO1* at 48 hpf and by *hsd3b2 MO2* at 33 hpf. C, Hsd3b enzymatic activity was inhibited by *hsd3b1MO1* but not by *hsd3b2MO2* at 50 hpf. In situ hybridization with *ff1b* probe shows the locations of the interrenal glands.

developed normally, whereas embryos injected with *hsd3b1*MO1 showed expanded *pomc* transcripts in the anterior pituitary at 2 dpf, and the expansion became more evident at 3 dpf (Figure 6). Quantitative real-time polymerase chain reaction analysis also showed increased *pomc* mRNA in embryos injected with *hsd3b1* MO (Figure 6G). At 4.5 dpf, control fish had normal pigmentation at 4.5 dpf (Figure 6E); however, animals treated with *hsd3b1*MO1 had more dispersed melanocytes (Figure 6F). The increased *pomc* expression and hyper-pigmentation are often found in patients with HSD3B2 deficiency (3). Thus, the *hsd3b1*MO1 phenotypes resemble those in human HSD3B2 deficiency.

HSD3B2-deficient patients have congenital adrenal hyperplasia due to excessive ACTH secretion (3). Therefore, we also examined *hsd3b1*(Dre9) morphants, which have reduced Hsd3b activity (Figure 7A). Cortisol was detected in 5-day fish injected with control MO, but the amount of cortisol was reduced after zebrafish was injected with *hsd3b1*MO1. This result indicated that depletion of *hsd3b1*(Dre9) resulted in insufficient cortisol secretion in these fish. Expression of the interrenal gland marker gene *ff1b* was increased in the interrenal gland of *hsd3b1* morphants when compared with that in control embryos (Figure 7). But the *ff1b* domain was diminished in the hypothalamus (Figure 7). This result indicates that *hsd3b1* morphants had interrenal hyperplasia and mimicked the phenotype of patients with HSD3B2 deficiency.

hsd3b2(Dre 20) knockdown led to embryo elongation

The earliest morphological abnormality of *hsd3b2* MO2-injected embryos was the elongation of the embryo (Figure 8A). This abnormality started at 75% epiboly stage (8 hpf) and was most easily identified at the bud stage. The elongation of the embryo was featured by the increase of the ratio of their animal-vegetal axis to medial-lateral axis (Figure 8). To determine whether this embryonic phenotype was dependent specifically on *hsd3b2* activity, we coinjected 1-cell embryos with 200-pg synthetic capped *hsd3b2* mRNA. Results showed that the percentage of embryos with the elongation phenotype dropped. The overall animal-vegetal to medial-lateral ratio was also similar to that of uninjected control embryos (Figure 8B). These results show that *hsd3b2* participates in embryo morphogenesis, and its depletion results in embryo elongation.

Discussion

Here, we report analysis of the function, expression, and evolutionary relationships of zebrafish *hsd3b* genes. We

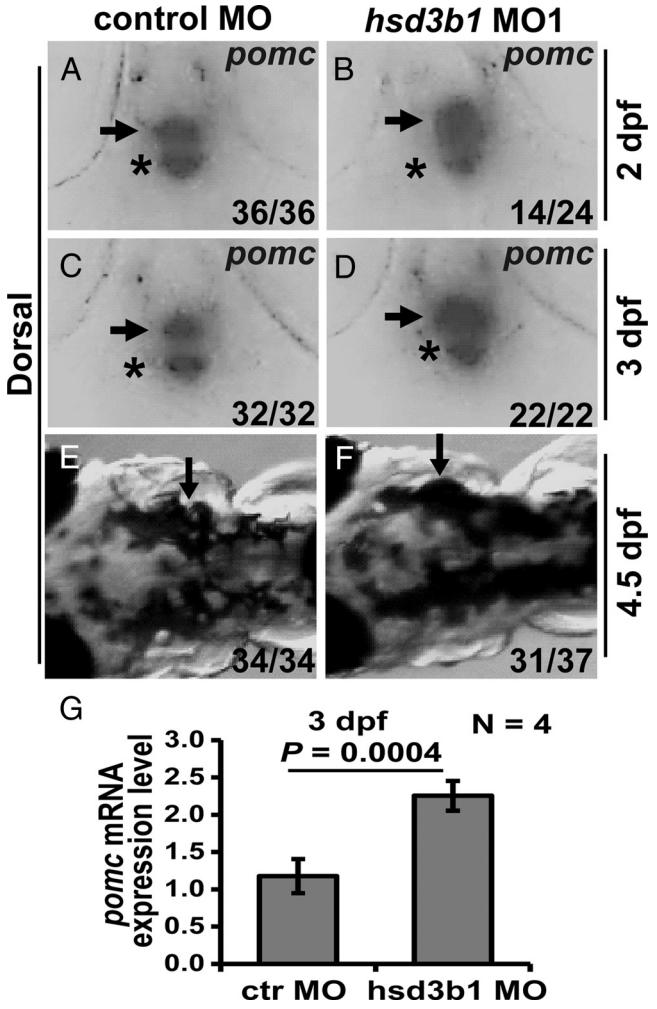

Figure 6. Depletion of *hsd3b1* results in increased *pomc* expression domains and larger areas of pigmentation. Zebrafish embryos were injected with (A and C) control MO or (B and D) *hsd3b1*MO1 followed by staining for (A–D) *pomc* at 50 hpf or by (E and F) direct examination of pigments at 4.5 dpf. Anterior is toward the top in A–D and toward the left in E and F. G, Quantitative real-time polymerase chain reaction analysis of *pomc* mRNA.

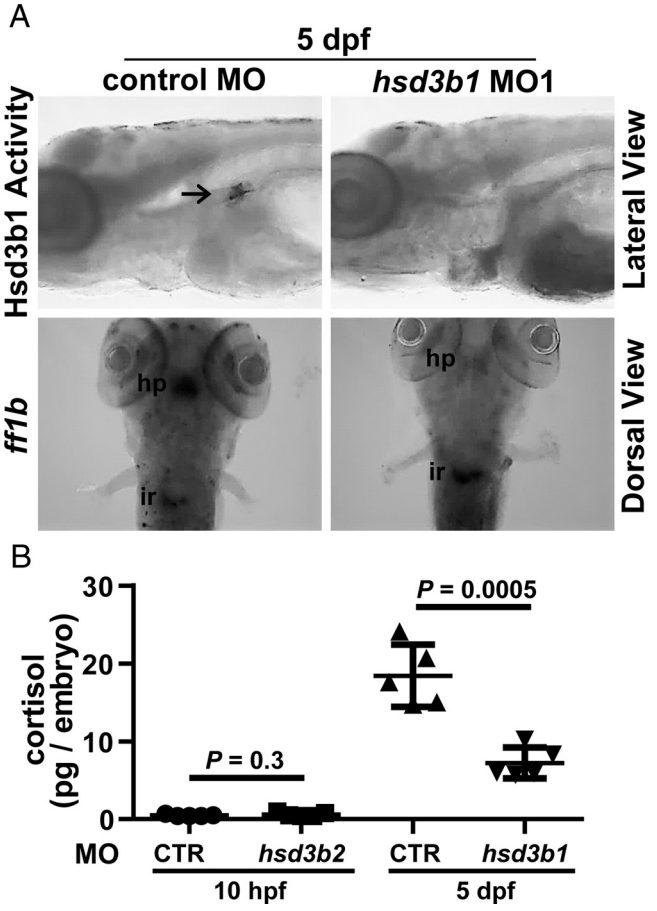

Figure 7. Depletion of *hsd3b1* diminished *ff1b* levels in the hypothalamus but enhanced *ff1b* levels in the interrenal gland. A, Hsd3b enzymatic activities were detected in control fish but were abolished by *hsd3b1*MO1. Zebrafish embryos were injected with control MO or *hsd3b1*MO1 followed by staining for *ff1b*. All larvae were examined at 5 dpf. B, Cortisol amounts in 5-dpf fish were reduced by *hsd3b1*MO1. The amount of cortisol in 10-hpf fish was low both with and without *hsd3b2*MO2 injection.

show that the 2 zebrafish paralogs are located on different chromosomes and are expressed at developmental stages. Although *hsd3b2*(Dre20) appears to perform embryonic functions important for embryo morphogenesis, *hsd3b1*(Dre9) is a mammalian *HSD3B* ortholog that is expressed in the interrenal glands and is important for endocrine functions. Thus, these 2 zebrafish genes appear to exert different physiological functions through expression at different developmental stages.

Evolution of *hsd3b* genes

In our phylogenetic analysis, zebrafish *hsd3b1* and *hsd3b2* group as sisters, indicating that these two genes arose via gene duplication after its ancestor diverged from the mammalian species. The mammalian isotypes then diverged after their own speciation. Therefore, these mammalian isoforms are more closely related to each other than to their paralogs from other species. This hypothesis is consistent with the data that zebrafish *hsd3b1*(Dre 9) appears to be the coortholog of Hsa *HSD3B1/HSD3B2*, or in other words, *hsd3b1*(Dre9) is equally orthologous to *HSD3B1* and *HSD3B2*. The *hsd3b2*(Dre 20) gene, on the contrary, is orthologous to neither human gene. Zebrafish is *hsd3b2*(Dre 20) located on a chromosomal segment unrelated to the human *HSD3B1/HSD3B2* region, also indicating that zebrafish *hsd3b* gene duplication occurs earlier than mammalian *HSD3B* gene duplication.

HSD3B nomenclature is a problem. The adult form (alias si:rp71-68n21.10, on zebrafish chromosome Dre9) was previously called *hsd3b2* because it was cloned later. Yet in this report, our phylogenetic and synteny analyses indicate that the adult form is the human *HSD3B1* ortholog, so it was renamed *hsd3b1*. The embryonic form (alias cb723 and zgc:122972, Dre20), which was previ-

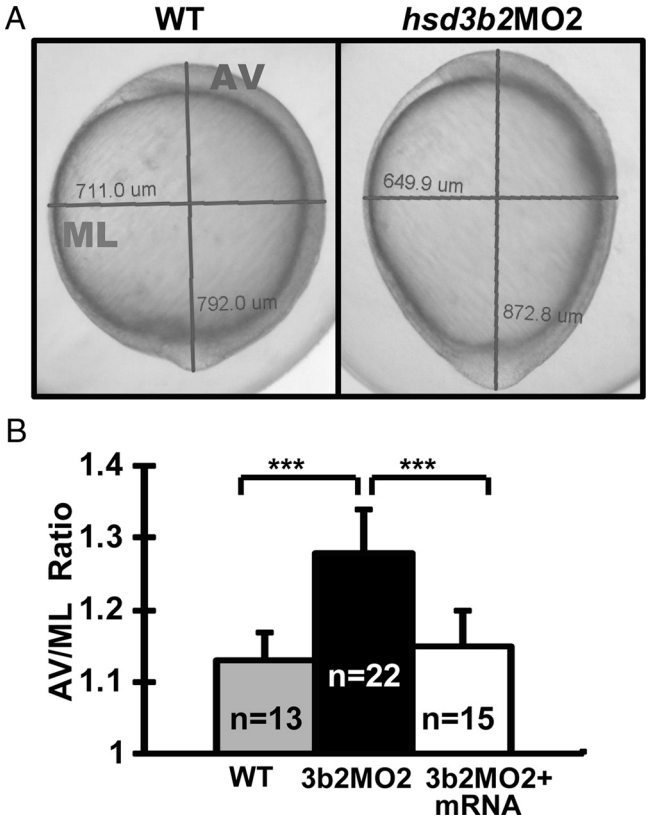

Figure 8. Blocking embryonic *hsd3b2* results in defective morphogenesis. Zebrafish embryos were injected with control MO or *hsd3b2*MO2, and the embryo dimension was measured. A, Zebrafish embryos at bud stage. AV, animal pole to vegetal pole axis; ML, medial-lateral axis. B, Injection of *hsd3b2*MO2 increases the AV/ML ratio, whereas coinjection with *hsd3b2* mRNA rescued the defect.

ously called *hsd3b1*, was renamed *hsd3b2* after consulting the zebrafish nomenclature committee (http://zfin.org/action/marker/view/ZDB-GENE-030828-2). In this report we have appended the chromosome number to both *hsd3b* genes so that they can be more readily distinguished.

Two *Hsd3b* genes differ in physiological functions

Zebrafish *hsd3b* genes could be distinguished roughly by their expression timing as the embryonic *hsd3b2*(Dre 20) and the adult *hsd3b1*(Dre 9). The *hsd3b1*(Dre9) gene was detected in adult interrenal glands and gonads mimicking the expression pattern of human *HSD3B2*. Its deficiency led to expanded interrenal glands, increased pigmentation, and enhanced *pomc* expression in the pituitary. These phenotypes are similar to congenital adrenal hyperplasia caused by *HSD3B2* deficiency in humans (3). Thus, the expression pattern, genomic location, and knockdown phenotype suggest that zebrafish *hsd3b1*MO1 knockdown animals can serve as a good model for the pathophysiological studies of human congenital adrenal hyperplasia caused by HSD3B deficiency.

The embryonically expressed *hsd3b2*(Dre20) gene, as shown in the present report, had a role in embryonic morphogenesis. Unlike mammalian embryos, which can obtain steroids from pregnant mothers through the placenta to support embryo development, zebrafish embryos rely on steroids present in the egg or synthesized by zygotic gene expression because zebrafish are oviparous. Therefore, zebrafish may have evolved a set of embryonic genes to support embryogenesis. It will be interesting to find out whether other oviparous fish also have a different *hsd3b* for embryonic development.

The embryos devoid of *hsd3b2*(Dre20) were elongated. Embryos become elongated when there is a defect in morphogenetic movements such as convergence and extension during gastrulation (19). Convergence and extension are regulated by a bone morphogenetic protein activity gradient (19). Mutation of *smad5* in the bone morphogenetic protein signaling pathway results in embryo elongation due to the defect of convergent extension movements (19). The phenotype of zebrafish *hsd3b2*(Dre20) knockdown suggests that *hsd3b2*(Dre 20) may encode an enzyme that produces a product that also helps control of zebrafish morphogenesis by convergence/extension, an important step in embryogenesis.

Role of Hsd3b in the HPI axis

We detected increased *pomc*-expressing corticotrophs of the anterior pituitary and reduced interrenal primordia at 50 hpf of *hsd3b1* MO-treated animals. The negative feedback loop of HPI axis in zebrafish is already active at 2 dpf (7). However, steroidogenic gene expression in the interrenal gland is only sensitive to pituitary regulation starting at 3 dpf (7). We also detected an increase in the interrenal domain at 5 dpf. The increased interrenal domain at 5 dpf is probably the consequence of the disruption of the pituitary regulatory negative feedback loop. Therefore, our data also suggested that glucocorticoid feedback at the pituitary level precedes pituitary-dependent regulation.

Summary

We have compared 2 zebrafish *hsd3b* genes. These 2 genes were located in chromosome 9 and chromosome 20, respectively. The gene located on chromosome 9, *hsd3b1*(Dre 9), is orthologous to human *HSD3B1/HSD3B2*. This gene was expressed in zebrafish interrenal glands, the counterpart of mammalian adrenal glands, and was important for the production of cortisol. Knocking down this interrenal Hsd3b resulted in the reduction of cortisol production, increased *pomc* expression in the hypothalamus, and hyperpigmentation. These symptoms were similar to those of human *HSD3B2* deficiency.

The embryonic *hsd3b2*(Dre20) gene was expressed as a maternal transcript in the embryonic blastomeres; its transcript was gradually reduced after gastrulation. This gene contributed to the morphological appearance of the embryo. It is located in a chromosomal region unrelated to human *HSD3B* genes.

Both zebrafish *hsd3b* genes encode functional enzymes, but they differ in their chromosomal location, expression pattern, and physiological functions. The *hsd3b1*(Dre 9) gene is orthologous to human *HSD3B* genes, whereas *hsd3b2*(Dre20) acquired a novel function. Our results provide insights into the evolution and the resulting consequences of the *hsd3b* genes.

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