Intrinsic Expression of a Multiexon Type 3 Deiodinase Gene Controls Zebrafish Embryo Size


State Key Laboratory of Medical Genomics (C.G., X.C., H.S.), Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, 200025 China; Thyroid Program of the Division of Endocrinology (C.G., X.C., H.S., M.A.M., R.H.J., N.Y.L., L.A.C., S.A.H.) and Clinical Research Center (H.A.F.), Boston Children’s Hospital; Stem Cell Program and Division of Hematology/Oncology (Y.Z., J.J.G., D.W., L.I.Z.), Boston Children’s Hospital, Harvard Stem Cell Institute, Harvard Medical School, and Howard Hughes Medical Institute; Department of Medicine (A.V.L., V.N.G., S.A.H.), Brigham and Women’s Hospital; Dana Farber Cancer Institute (V.N.G., L.I.Z., S.A.H.), Boston, Massachusetts 02115; and Departments of Physiology and Pharmacology (T.S.S.), Oregon Health and Science University, Portland, Oregon 97239

Thyroid hormone is a master regulator of differentiation and growth, and its action is terminated by the enzymatic removal of an inner-ring iodine catalyzed by the selenoenzyme type 3 deiodinase (dio3). Our studies of the zebrafish reveal that the dio3 gene is duplicated in this species and that embryonic deiodination is an important determinant of embryo size. Although both dio3 paralogs encode enzymatically active proteins with high affinity for thyroid hormones, their anatomic patterns of expression are markedly divergent and only embryos with knockdown of dio3b, a biallelically expressed selenoenzyme expressed in the developing central nervous system, manifest severe thyroid hormone-dependent growth restriction at 72 hours post fertilization. This indicates that the embryonic deficiency of dio3, once considered only a placental enzyme, causes microsomia independently of placental physiology and raises the intriguing possibility that fetal abnormalities in human deiodination may present as intrauterine growth retardation. By mapping the gene structures and enzymatic properties of all four zebrafish deiodinases, we also identify dio3b as the first multiexon dio3 gene, containing a large intron separating its open reading frame from its selenocysteine insertion sequence (SECIS) element. (Endocrinology 155: 4069–4080, 2014)

Thyroid hormone is a master regulator of cellular metabolism and growth. In adults, thyroid hormone signaling regulates energy expenditure, cardiac function, and fluid homeostasis. In children, thyroid hormone also mediates the proliferation and differentiation of growing tissues. The latter functions have been demonstrated in various animal models and are also illustrated in humans by the short stature and mental retardation that result from inadequately treated congenital hypothyroidism or fetal Graves’ disease (1, 2).

Most thyroid hormone secreted from the iodine-sufficient gland is in the form of the prohormone 3,5,3′,5′-thyroxine (T4) that is metabolized in peripheral tissues by the iodothyronine deiodinase family of selenoenzymes. In all vertebrates studied to date, type 2 deiodinase (D2) activates T4 to the more potent 3,5,3′-triiodothyronine (T3) by outer-ring deiodination. Conversely, the type 3 deiodinase (D3) inactivates both T4 and T3 by removal of one or both inner-ring iodines. The products of this inner-ring deiodination, 3,3′,5′-rT3 and 3,3′-T2, are unable to bind...
the thyroid hormone receptor and are thus biologically inactive. Type 1 deiodinase (D1) is unique in its capacity to catalyze both the outer-ring deiodination of T4 and the inner-ring deiodination of T4 and T3 (3).

Orthologs of the mammalian deiodinases have been identified in fish by sequence homology. Early zebrafish studies have indicated that D2 regulates the tempo of early embryonic development (4, 5) and D3 has been implicated in the process of fin regeneration (6). Of note, whereas these reports acknowledged three zebrafish deiodinase genes (dio1 on chromosome 8, dio2 on chromosome 17, and dio3 on chromosome 20), a second dio3 gene on chromosome 17 was previously proposed in 2000 (7) and has recently been confirmed in the completed zebrafish genome (8). In adherence to recommended nomenclature, recently been confirmed in the completed zebrafish genome (8). In adherence to recommended nomenclature, this earliest reported gene has been renamed dio3a and the chromosome 20 gene renamed dio3b. Heijlen et al (9, 10) has recently shown that morpholino-mediated knockdown of either dio3a or dio3b results in partial loss of inner-ring deiodinase activity in the whole embryo, indicating that both paralogs may encode active enzymes. However, the enzymatic properties and detailed anatomic expression of these zebrafish deiodinases have not been experimentally defined.

Our goals were to determine whether or not both dio3 gene(s) are functional and, if so, whether they have similar or different roles during development. Our present study shows that both zebrafish dio3 paralogs are syntenic to human DIO3 and that in in vitro studies each encodes a functional selenoenzyme that potently inactivates both T4 and T3. Although the enzyme kinetics of both zebrafish Dio3 proteins are similar, their expression patterns and gene structures are markedly divergent and we report here that dio3b is the first D3 gene from any species to possess a large intron separating its open frame from its selenocysteine insertion sequence (SECIS) element, a critical 3’UTR sequence required for selenoprotein synthesis, selenocysteine insertion at UGA codons, and deiodinase catalytic activity. Consistent with paralog subfunctionalization, we observed severe microsomia with knockdown of dio3b but not dio3a. This provides direct evidence that embryonic D3 activity is critical for the determination of fetal size and suggests the possibility that human deficiencies in fetal DIO3 expression could present as intrauterine growth retardation.

**Materials and Methods**

**Fish embryos and embryonic treatments**

Wild-type embryos from the Oregon AB line were maintained under standard conditions at 28.5°C and pH 8.0. In some embryo experiments, dronedarone (10 μM) or vehicle were added to the fish water. Embryo water was changed every 1 to 2 days. T3 treatment of other embryos was performed by the method of Heijlen et al (9), using Danieau’s medium supplemented with 0 versus 50 nM T3. All experiments were approved by our institution’s animal care and use committee. All reagents were purchased from Sigma-Aldrich.

**In silico analysis**

Sequences with homology to human DIO3 were identified in the National Center for Biotechnology Information (NCBI) Genbank and the University of California Santa Cruz databases. The NCBI Blast was used to compare the homology of predicted peptide sequences. SECISeach analysis (11) was used to predict SECIS elements.

**3’-Rapid amplification of cDNA ends (3’RACE)**

Zebrafish tissues were surgically dissected and snap frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen) and mRNA prepared with the Oligotex mRNA mini kit (Qiagen). The 3’ RACE library was constructed with the GeneRacer system (Invitrogen), using an oligo-dT primer and forward gene-specific primers based on the partial dio3b sequence in Genbank (NM_001177935.2). The sequences of our nested gene-specific primers were 5’-ACACAGATGG TCA CGCTGGAGTGC-3’ and 5’-GGAGACCCTGATCCTCAA CTTC-3’. Amplified products were cloned into the pGEM-T Easy vector system (Promega) for sequencing.

**Imprinting analysis**

DNA from individual adult fish was genotyped with primers flanking the dio3b polymorphism: 5’-TCTAGGAGACCCCATGGCAGCTGA-3’ (forward primer); 5’-AGAGGAAGAAAGCG TCTGTGCG-3’ (reverse primer). Total RNA was prepared from each fish’s surgically dissected tissues as described above, digested with DNase I, and reverse transcribed. PCR was then performed on these templates with nested primers flanking dio3b’s polymorphism. The sequences of our nested primers were: 5’-GACGTCGCTAGGACCCCATGGC-3’ (outer forward primer); 5’-TCTAGGAGACCCCATGGCAGCTGA-3’ (inner forward primer); 5’-AACCGACTGTCGACACAGCAT-3’ (outer reverse primer); 5’-ATCTGATCCTGATCAGGCTTG-3’ (inner reverse primer).

**Generation of pSelExpress constructs**

To test the function of putative SECIS elements, we removed the SECIS element from pSelExpress1 (12) and designated the modified vector pSelExpress-minus. The full-length dio3b cDNA we identified by 3’-RACE was cloned into this pSelExpress-minus vector for transient transfection experiments and then mutated for the experiments in Figure 1D. Zebrafish dio1, dio2, and dio3a cDNAs were purchased from Open Biosystems and the open reading frame (ORF) of each cloned into pSelExpress-minus vectors or into SECIS-containing vectors for transient transfection and in vitro enzyme studies.

**Tissue Culture and transient transfection**

Human embryonic kidney (HEK-293) epithelial cells were propagated in DMEM media with 10% FBS and 100 nM sodium selenite. Transient transfection was performed with Lipo-
fectamine Plus reagents (Invitrogen) according to the manufacturer’s instructions. A pCMV<HUB><HSP>β-gal plasmid was used to control for transfection efficiency (13). Unless stated otherwise, all chemical reagents were purchased from Sigma-Aldrich.

Deiodination assays

Cellular sonicates were prepared in 0.1 M phosphate and 1 mM EDTA at pH 6.9 with 10 mM dithiothreitol and 0.25 M sucrose. Deiodinase activity was assayed as previously described (14) using 0 to 150 µg of cellular protein, and 50,000 to 200,000 cpm of 3,5,3'<sup>[125I]</sup>T4; 3,5,3'<sup>[125I]</sup>T3; or 3,3'<sup>[125I]</sup>rT3 (Perkin Elmer). Human deiodinases were incubated at 37°C and zebrafish deiodinases were incubated to 28.5°C. Reactions were stopped by the addition of methanol, and the products of deiodination quantified by HPLC. D3-specific activities are expressed as fmol of T3 inner-ring deiodinated per mg of sonicate protein per minute (fmol/mg/min). When appropriate, β-gal reporter expression was used to control for transfection efficiency. For calculations of Km by Lineweaver Burk analysis, substrate concentrations ranged from 0.3 to 8.0 nM for T4 and from 0.5 to 8.0 nM for T3.

Western blotting

Cellular sonicates were prepared from transfected HEK cells as described above. Thirty µg of each sonicate were resolved on a 12% Tris-HCl gel and transferred to PVDF membrane with the BioRad Semi-Dry system. The BM Chemiluminescence Western Blotting Kit (Roche) and 1:5000 monoclonal anti-Flag M2 antibody (Sigma) were used according to the manufacturers’ instructions.

Morpholinos, microinjection, and whole-mount in situ hybridization

Morpholinos were purchased from Gene Tools, LLC. Embryos were collected 20 minutes after fertilization for microinjection at the 1 cell stage. Microinjection was performed by standard methods (15), using 1 to 1.4 nL injection volumes and 2.8 ng of morpholino per embryo. The morpholino sequences are as follows: dio3a-MO1, 5'<sup>G</sup>-GCGACGCTCCCGATTCAGACATCAAGTCGCGG-3'; dio3b-MO1, 5'<sup>G</sup>-CTTCCCCTTACCTCATCAAGTCGCGG-3'; dio3a-MO2, 5'<sup>G</sup>-TGGTTGTCTTACCTCCTTTACCCATCCGT-3'; dio3b-MO2, 5'<sup>G</sup>-GGTGGTCTTACCTCCTTTACCCATCCGT-3'; and control-MO, 5'<sup>G</sup>-CCTCTTACCTCAGTTACAATTTATA-3'.

Whole mount in situ hybridization was performed as previously described (16). Ten to 15 embryos from each time point were combined and hybridized with digoxigenin-labeled anti-
sense RNA probes at 68°C. Proteinase K was used for embryos at 72 hours post fertilization (hpf) or older. All bright-field images were acquired at the same magnification, exposure, and gain. For measurements of length, embryos were fixed in paraformaldehyde at 72 hpf (22–92 embryos measured per group). Embryos were measured using ImageJ software and the body axis length of morphant embryos is shown as a percentage of the average length of control embryos.

**Gene expression**

Total RNA was prepared from pooled whole embryos using Trizol (Ambion) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantification of mRNA was performed by the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with SYBR Green as the detector dye. The β-2-microglobulin gene was used as an internal control. Primers sequences are available upon request.

**Statistical analysis**

The analysis of enzyme activity in mutant Dio3b proteins was performed by 1-way ANOVA adjusted for inhomogeneous variance (17). Further comparisons between the mutants and empty vector were adjusted to maintain the familywise Type I error rate (probability of any false findings of significant difference) below 5%, by two different methods: Dunnett’s procedure for comparing several groups to a common control (18) and hierarchical testing of a closed family (19). Both analytic methods indicated significantly higher activity for the cysteine mutant compared with empty vector (P < .0001), but no difference for the other mutants. The analysis of relative D3 activity was performed by 1-way ANOVA and the analysis of embryo length was performed by 2-way ANOVA. Comparisons between T3 and vehicle-treated embryos were performed by unpaired t test. P values less than .05 were considered significant.

**Results**

Two zebrafish dio3 genes with disparate structures encode active selenoenzymes

Recent analysis of the full zebrafish genome has revealed two putative dio3 genes, dio3a and dio3b (8). Comparing their predicted amino acid sequences, both are > 60% identical with the human D3 protein and they are 77% identical with one another. Gene duplication is a well-established phenomenon in fish that can occur through multiple mechanisms, including gene retrotransposition, tandem duplication, and whole genome duplication (20). To address these possibilities, we performed an in silico analysis that shows that the 5Mb genomic sequences flanking the human and zebrafish dio3 orthologs contain multiple shared coding genes (Figure 1A). This indicates that both zebrafish genes are syntenic to human DIO3. We further found that the genomes of several other fish species possess two rather than one predicted gene product with high homology (>60% identical) to human DIO3 (Supplemental Table 1) (8, 21). Collectively, these data support that dio3a and dio3b are paralogs that arose from the polyploidization event near the base of ray-finned fish evolution (22).

We next compared the gene structures of dio3a and dio3b. All iodothyronine deiodinases are selenoproteins that require incorporation of the 21st amino acid selenocysteine (Sec) for enzyme activity. This selenoprotein synthesis requires both an in-frame TGA within the ORF and a cis-acting RNA factor in the 3’UTR called the SECIS element. SECIS elements form a unique stem-loop structure that binds SECIS-binding protein 2 (SBP2) and recodes in-frame TGA codons to specify Sec rather than stop (23, 24). SECIS-binding protein 2 in turn recruits the Sec tRNA-specific elongation factor, EFsec, bound to tRNA^Sec that is required for all eukaryotic selenoprotein synthesis. Of note, in all species studies to date, the structure of dio3 genes is conserved and comprised of a single exon that contains both the ORF and the SECIS element (8).

Using the SECISearch computational tool that recognizes SECIS elements on the basis of predicted structural and thermodynamic features (11), the zebrafish dio1, dio2, and dio3a mRNA sequences in the NCBI database were confirmed to contain strong SECIS elements. However, to our surprise, no SECIS element was recognized for dio3b. This suggested that dio3b was either a pseudogene that lost SECIS function and enzyme activity due to paralog subfunctionalization, or that its gene structure was novel. To address the latter possibility, we performed 3’ rapid amplification of cDNA ends (3’-RACE) using mRNA prepared from pooled zebrafish brains. This identified a novel full-length dio3b transcript that, when compared with the genomic sequence, is encoded by two exons separated by a 5,398 bp intron that is flanked by classic GT-AG dinucleotide consensus splicing sequences. The first dio3b exon contains the ORF and the second exon contains a 103 bp sequence predicted to be a strong SECIS element by SECISearch analysis, indicating that dio3b’s gene structure is distinct from all previously cloned D3 genes (which are intronless; Figure 1B).

To test the function of this putative SECIS and to assess the dependence of Dio3b enzyme activity on Sec incorporation, we generated a series of mutant constructs (Figure 1C). The first mutation (stop mt) converts the in-frame TGA within the dio3b ORF into a TAA stop codon. The cysteine mt and leucine mt mutations convert this TGA codon to cysteine (Cys) or leucine (Leu), respectively. Finally, the SECISmt introduces two point mutations into the SECIS stem to disrupt stem-loop formation and SECIS function. All mutants were Flag-tagged at the amino terminus and transiently transfected into HEK cells. Western blot analysis of the resultant cell sonicates showed that the
wild-type dio3b plasmid produced both a 16 kDa protein (corresponding to termination at the in-frame TGA) and a 31 kDa protein (corresponding to read-through across the in-frame TGA and termination at the downstream TAG stop codon). Consistent with SECIS-mediated Sec incorporation, the full-length wild-type Dio3b protein was equal in size to the cysteine and leucine mutants and the truncated wild-type Dio3b protein comigrated with the stop and SECISmt mutants (Figure 1D). Of note, this translation of both truncated and full-length proteins from the wild-type dio3b sequence is typical of transiently overexpressed selenoproteins, including the human deiodinases (24).

Enzyme activity assays performed on these same sonicates showed that SECIS incorporation is necessary for Dio3b enzyme activity (Figure 1E). Strong T3 inner-ring deiodinase activity was present only in the wild-type dio3b protein product. Substituting the structural analog Cys for Sec reduced specific enzyme activity 49-fold, similar to the 10–100-fold lower enzyme efficiencies characteristic of the Sec-to-Cys mutants of other selenoenzymes (24, 25). No deiodinase activity was measurable in the stop, leucine, or SECISmt mutant proteins. As final proof that the putative dio3b SECIS is functional, we generated additional mutants containing the ORF of zebrafish dio1 or dio2, either alone or subcloned upstream to the minimal dio3b SECIS. In both cases, addition of the dio3b SECIS produced strong substrate-specific deiodinase activity (Figure 1F), conferring rT3 outer-ring deiodinase activity to the dio1 ORF and T4 outer-ring deiodinase activity to dio2. Collectively, these data document that dio3b is an authentic selenoenzyme that requires both SECIS function and Sec incorporation for enzyme activity. In addition, the dio3b gene structure is distinct from all previously cloned dio3 genes as the first to contain an intron.

**Enzyme kinetics and divergent expression of the zebrafish dio3 genes**

Dio3a and dio3b offer an unprecedented opportunity to compare the function of two paralogous deiodinases. Extensive mammalian studies have illustrated that the enzymatic properties of deiodinase proteins cannot be fully predicted on the basis of sequence homology (26). Thus, we designed a transient transfection system to directly study iodothyronine catalysis in vitro. The ORF of each zebrafish deiodinase was cloned into a vector containing both the SECIS-binding protein 2 gene (to optimize selenocysteine incorporation) and a highly efficient SECIS element engineered to optimally express selenoproteins in mammalian cells (12). These plasmids were transfected into HEK cells that possess negligible endogenous deiodinase activity, and the enzyme activity of the resultant sonicates assayed by incubation with radiolabeled iodothyronines. The low endogenous deiodinase expression of HEK cells has been characterized in prior reports (26) and was confirmed in the current studies by documenting the lack of substrate conversion in sonicates prepared from cells that were untransfected or transfected with only empty vector.

Enzymatic assays documented that both Dio3a and Dio3b are potent inactivators of thyroid hormone. Lineweaver Burk analysis confirmed extremely high substrate affinity that, like human D3, is characterized by low nanomolar Km’s for both the T4 prohormone (Figure 2A) and the more active T3 (Figure 2B). This indicated that one or both dio3 gene product could inactivate thyroid hormone, as is the case in mammals. Despite these similarities, extended functional studies revealed differences in other substrate affinities, including the surprising ability of Dio3a to outer-ring deiodinate rT3, a feature that is lacking in both Dio3b and in human D3 (Figure 2C).

In mammals, Dio3 is paternally imprinted (27). To determine if this epigenetic regulation is relevant in the zebrafish, we took advantage of a common polymorphism we identified within the 3’UTR of dio3b, defined by a 20 nucleotide deletion (TTGAGTGATTGATTGAGTGA) 40 bp downstream of the ORF. Of note, this deletion does not alter the SECIS element or enzyme activity (data not shown). To assess imprinting, RNA was isolated from the dissected brain, liver, and fin tissues of individual adult fish and then reverse transcribed to prepare cDNA. Using nested PCR primers that flank the 3’UTR deletion, two dio3b amplicons of appropriate size were documented in fish heterozygous for the polymorphism (Fish 2 and Fish 3 in Figure 2D), indicating biallelic rather than imprinted expression.

Deiodination is unique in its ability to produce tissue-specific and temporally rapid changes in local thyroid hormone signaling (28, 29). Thus, despite the above data showing nearly identical T4 and T3 catalysis by Dio3a and Dio3b, their in vivo functions could differ dramatically due to different expression patterns. To assess this, we performed in situ hybridization (Figure 2E) in embryos through 5 days post fertilization (dpf). At 24 hpf, both dio3a and dio3b signal was visible around the yolk surface. In addition, specific dio3a expression was present in the hindbrain and otic vesicles at early time points and then decreased after 2 dpf. For dio3b, strong expression was present throughout the forebrain, midbrain, and hindbrain at 24 hpf and broad expression in the brain was noted at 48 hpf and 5 dpf. Specific dio3b expression was also present in the pronephric ducts, the retina, and in
gastrointestinal structures, including the liver. In summary, dio3a and dio3b both encode enzymatically active proteins with similar kinetic properties to human D3. However, distinct functional roles can be predicted from their differing expression patterns and some aspects of gene regulation are fundamentally different from the mammalian ortholog, as illustrated by dio3b’s biallelic expression.

**Enzymatic properties of the zebrafish deiodinase family are divergent from mammals**

Prior zebrafish studies have operated on the assumption that their deiodinases catalyze the same enzymatic reactions as their mammalian counterparts. To test this, we extended our in vitro pSelExpress1 approach to all 4 zebrafish deiodinases. For each deiodinase, assay conditions were titrated to fully exhaust the enzyme’s primary iodothyronine substrate (rT3 for Dio1, T4 for Dio2, and T3 for Dio3a and Dio3b). These assay conditions were then held constant and alternative substrates substituted into parallel reactions (Figure 3A). This revealed important differences between mammalian and zebrafish deiodinase enzymes (Figure 3B), including the outer-ring deiodination of rT3 by zebrafish Dio3a. In addition, under the conditions tested, zebrafish Dio1 differed from its mammalian ortholog by showing minimal ability to outer-ring deiodinate T4 or to inner-ring deiodinate T3. Thus, in the zebrafish, D2 appears to be the primary activator of T4 and Dio3a/Dio3b appear to be the major inactivators of T3.

**Deficiency of Dio3b but not Dio3a reduces embryo size at 72 hpf**

Mice with global D3 deficiency suffer severe growth retardation and significant rates of perinatal death, indicating a critical role of D3 in fetal development (30). However, because this rodent model also causes placental D3 deficiency and subsequent fetal thyrotoxicosis from the increased transfer of maternal thyroid hormone (31), the specific role of embryonic (vs placental) D3 expression cannot be defined. Aquatic vertebrates provide the opportunity to avoid the confounders of placental metabolism and maternal thyroid status. To directly test the developmental function of embryonic D3, we used morpholino microinjection to inhibit the expression of dio3a or dio3b.
For each gene, two different morpholinos directed to nonoverlapping target sequences were used to confirm phenotype specificity. *Dio3a* morpholinos were designed to block translation (*dio3a*-MO1) or SECIS formation (*dio3a*-MO2). *Dio3b* morpholinos were designed to block translation (*dio3b*-MO1) or splicing (*dio3b*-MO2). After injection, enzyme assays were performed on pooled whole embryo sonicates to validate morpholino knockdown at the level of protein function. T3 inner-ring deiodination of 72 hpf embryos was decreased by 45% or 3% (*P* < .001) or by 22% or 5% (*P* = .031) after injection of a *dio3a* morpholino; and by 88% or 1% (*P* < .0001) or by 74% or 9% (*P* < .0001) after injection of a *dio3b* morpholino (mean +/- standard error), indicating that *dio3b* accounts for most D3 activity at this developmental stage (Figure 4A). Focusing on the body length phenotype at 72 hpf, knockdown of *dio3a* produced no obvious abnormalities when compared with embryos injected with a standard control morpholino. In contrast, knockdown of *dio3b* dramatically shortened body length to 81% of normal (*P* < .0001, Figure 4, B and C). Because the established physiologic role of D3 is to inactivate T3, we hypothesized that the growth restriction in *dio3b*-deficient embryos is caused by the abnormal elevation of thyroid hormone signaling in tissues that normally express this paralog. To test this, we treated *dio3b*-deficient embryos with the thyroid hormone antagonist dronedarone (32). Dronedarone significantly ameliorated the growth failure (*P* < .05), with a 4% to 6% increase in length compared with the vehicle group (Figure 4C), indicating that this pathophysiology is indeed thyroid hormone-dependent.

Figure 3. Substrate Specificity of the Zebrafish Deiodinas. (A) Individual zebrafish deiodinas were overexpressed and incubated with 125I-radiolabeled iodothyronines (T4, T3, or rT3) to assess substrate specificity. (B) Comparison of the zebrafish pathways revealed in (A) to known human deiodination pathways.
Figure 4. Embryonic Microsomia from dio3b Knockdown  
(A) Whole embryo D3 enzyme activity is decreased after morpholino-mediated knockdown of Dio3a (**, \(P < .001\) or *, \(P < .05\) compared with control morpholino) or Dio3b (**, \(P < .0001\) compared with control morpholino). The average of multiple experiments is shown, using embryos that were singly-injected with either control morpholino (n = 5), dio3a-MO1 (n = 4), dio3a-MO2 (n = 4), dio3b-MO1 (n = 5), or dio3b-MO2 (n = 4). Enzyme activity was measured at 72 hpf.  
(B) At 72 hpf, embryos injected at the 1-cell stage with morpholinos to knockdown dio3a are normal in size whereas embryos injected with morpholinos against dio3b are microsomic. Scale bar is 0.5 mm.  
(C) Quantitative analysis of body length confirms significant loss of length (**, * \(P < .0001\)) compared with standard control morpholino after Dio3b knockdown (either dio3b-MO1 or dio3b-MO2 reduce length by approximately 19%) but not Dio3a knockdown (no significant reduction in length with either dio3a-MO1 or dio3a-MO2). The thyroid hormone antagonist dronedarone (10 \(\mu\)M) confers partial but significant rescue of body length (*, \(P < .05\)).  
(D) The whole embryo D3 enzyme activity of uninjected embryos is increased 2.5-fold by exposure to 50 nM T3 (left panel; **, \(P < .001\)). This is accompanied by a 2.0-fold increase in dio3a mRNA (middle panel; **, \(P < .001\)) and a 2.8-fold increase in dio3b mRNA (right panel; **, \(P < .001\)).
**Dio3a and dio3b are induced by thyroid hormone**

Exposure to high levels of exogenous T3 has been shown to partially mimic the phenotype of embryonic D3 deficiency in both mice (30) and zebrafish (9). Because D3 expression has been shown to increase after T3 treatment in adult rats (33), we hypothesized that the discordance between the developmental phenotypes of T3 excess and D3 deficiency is partially due to compensatory induction of D3 in response to thyrotoxicosis. To test this, we treated uninjected embryos with 50 nM T3 vs. vehicle. At 3 dpf, T3-treated embryos displayed markedly induced D3 activity (2.5-fold, \( P < .001 \), dio3a mRNA (2.0-fold, \( P < .001 \)), and dio3b mRNA (2.8-fold, \( P < .001 \)) when compared with the vehicle group (Figure 4D), indicating that both D3 paralogs are positively regulated by thyroid hormone during embryonic development.

**Discussion**

Thyroid hormone is a master regulator of cellular differentiation and growth, and deiodination is the major pathway of thyroid hormone metabolism. Given this, it is not surprising that the deiodinases are members of the ancestral selenoproteome, a group of only 21 selenoproteins that are conserved across all vertebrates (8). D3 activity was first discovered in mammals and its best-characterized function is as a placental enzyme that shields the developing fetus from maternal thyroid hormone (34, 35). However, its evolutionary conservation implies other important functions that predate the origin of mammals and even the colonization of terrestrial environments. Insight into these primordial functions can be gained through studies of aquatic vertebrates, illustrated by the seminal Xenopus experiments that revealed the dependence of amphibian metamorphosis on thyroid hormone (36). Here, through investigations of a duplicated dio3 gene, we have identified an important developmental role of its embryonic expression in the determination of embryo size and have discovered the first multiexon dio3 gene, dio3b.

Recent analyses of EST and genomic databases have revealed that dio3 is duplicated in several teleost species. We chose to study this duplication in the zebrafish because its genome is completely sequenced and to investigate its potential as an animal model to study the role of deiodination in development and tissue injury. Our analysis revealed that both zebrafish paralogs are syntenic to human Dio3 and that each encodes an active enzyme with potent inner-ring deiodinase activity. Interestingly, despite nearly identical enzyme kinetics with regard to T4 and T3, their tissue expression patterns are markedly distinct. Collectively, the sum of dio3a’s and dio3b’s tissue expression approximates that of the single mammalian Dio3 gene, suggesting that each zebrafish gene lost a portion of its expression through paralog subfunctionalization. Unlike other Dio3 genes, zebrafish dio3b is biallelically expressed, supporting the recent hypothesis that imprinting of the Dlk1-Dio3 cluster evolved specifically in the eutherian domain (37). Further work will be necessary to determine if dio3a is imprinted and if dio3b may be imprinted in other tissues or at other developmental time points.

The most striking feature of the dio3b gene structure is a large 5,398 bp intron that separates its ORF and SECIS element. This contrasts with all other known Dio3 genes that are intronless, and resembles the precedent of another selenoprotein, selenophosphate synthetase 2, that evolved into an intronless gene in early mammals by replacement of the original multiexon gene (8). Because Dio3 is paternally imprinted in placental mammals, our findings also follow the general trend that imprinted genes tend to have fewer and smaller introns (38). However, the hypothesis that the onset of dio3 imprinting in eutherian species drove intron loss is disproven by the absence of dio3 introns in other teleost species (Supplemental Table 1), which instead supports the opposite possibility of intron gain in the zebrafish. It is interesting to note that zebrafish dio3b’s gene structure is also atypical compared with other intron-containing selenoproteins because in-frame TGA codons and SECIS elements nearly always reside within a shared exon.

In all previously characterized species, D1 and D2 activate the T4 prohormone into the more potent T3 and, conversely, D3 is the major inactivator of both T4 and T3. Our in vitro studies confirm these pathways for zebrafish Dio2 and Dio3. However, to our surprise, our studies of zebrafish Dio1 showed minimal catalysis of either T4 or T3. This suggests that, in the zebrafish, Dio2 is the primary activator of T4 and the Dio3 enzymes are the major inactivators of both T4 and T3. This may explain the lack of developmental abnormalities recently reported by Walpita et al (4) after solitary D1 knock down. Of note, this contrasts other fish species such as tilapia where Dio1 is known to outer-ring deiodinate T4 (39) and suggests that zebrafish lost this Dio1 function as they evolved from other teleosts.

Further study is warranted to investigate if zebrafish Dio1 may function as an inactivator of conjugated (40) or deaminated (41) thyroid hormones and, given the recent demonstration of an amphibious deiodinase that functions optimally at only low temperatures (41), experiments to examine the enzymatic activity of zebrafish deiodinases under extended ranges of temperature and thiol cofactor concentration are also justified. Future studies should also
consider the role of deiodinases in the metabolism of other iodothyronines, such as 3,5-T2 that has been shown in recent studies to transactivate the TRβ1 receptor in teleosts (42). From a broad perspective, these findings demonstrate that the enzymatic function of some teleost deiodinases may differ from their mammalian orthologs despite overall high sequence homology, and underscore the value of directly testing their enzyme properties.

Our morpholino studies reveal that dio3b deficiency dramatically reduces embryo size. This finding resembles the 35% growth retardation observed in global D3 knockout mice (30), but is novel because it occurs in the absence of the potential effect of placental physiology. In humans, the placenta has the highest D3 activity of any normal tissue and this defines D3’s established physiologic role as a biochemical shield that protects the developing fetus from the temporally inappropriate action of maternal thyroid hormone (34). D3 knockout mice lose >97% of their placental D3 activity (even in heterozygous mothers) and this alone is sufficient to explain their fetal thyrotoxicosis and perinatal dysmorphology. These confounders prevent the definitive assessment of embryonic D3’s function in rodent models. In contrast, the growth restriction in our zebrafish model is direct evidence that D3, once considered only a placental enzyme (43), can regulate embryo size by mechanisms that are independent of placental tissue and that embryonic D3 deficiency alone is sufficient to cause microsomia. Of note, our results differ somewhat from the recent report from Heijlen et al (9) that describes a transient decrease in body length in dio3a as well as in dio3b morphants. This discrepancy can be explained by our current study’s focus on earlier developmental time points.

Based upon measures of whole embryo D3 activity, morpholino-mediated knockdown was present from single cell injection through 72 hpf, which corresponds to the earliest stages of mammalian fetal development (when embryonic D3 expression is high) through the equivalent of the mammalian neonatal thyroid surge (generally considered analogous to teleost or avian hatching). Similar to the first trimester of human pregnancy, when fetal glandular secretion is negligible and T3 is provided exclusively by the mother, maternal thyroid hormone is supplied by the zebrafish yolk until hatching and activation of the larval thyroid gland. Of note, the presence of this maternal T4 and T3 at the start of zebrafish development has been directly documented by others via immunoassay (44). Further studies will be necessary to identify the mechanism(s) by which D3 controls embryo size, but the partial amelioration of microsomia in dio3b-deficient morphants by the thyroid hormone antagonist dromedarone indicates that the precise modulation of thyroid hormone receptor signaling in embryonic structures that normally express dio3b is required for optimal growth. Our in situ hybridization data indicate that the brain, the pronephros, and the gastrointestinal tract are potential candidates. We hypothesize that the derangement of thyroid hormone signaling in these tissues impairs local organ function and development in a manner that then causes downstream abnormalities and growth arrest in other non-D3 expressing tissues. Further experiments to investigate this hypothesis are warranted and may provide much needed insight into the effects of thyroid hormone excess on human growth, which commonly manifests as microsomia in the fetal/neonatal period but paradoxically accelerates linear growth in school-age children.

From a broad perspective, our findings indicate an important functional role for embryonic expression of D3 in development and support the appropriateness of zebrafish as a model to study thyroid hormone metabolism. Recent advancements in CRISPR/Cas system genome editing (45) will facilitate the generation of permanent zebrafish deiodinase knockout animals to complement conventional morpholino approaches. The finding that embryonic D3 is required for optimal body length indicates additional complexity in the numerous actions of thyroid hormone on growth and, from an evolutionary standpoint, supports the parental conflict hypothesis of genomic imprinting that states that paternally imprinted genes like Dio3 are selected for their ability to recruit maternal resources and enhance fetal size (46). Our data illustrate that the expression of dio3 paralogs is anatomically specific, temporally dynamic, and responsive to systemic thyroid status during even the earliest stages of embryonic development. From a clinical standpoint, our data also raise the intriguing possibility that even transient abnormalities in embryonic deiodination could present as intrauterine growth retardation in humans, a global pediatric health problem that is incompletely understood and increasingly linked in risks of adult disease (47).

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Address all correspondence and requests for reprints to: Stephen A. Huang, Children’s Hospital Boston, 300 Longwood Avenue, Boston, MA 02115. E-mail: stephen.huang@childrens.harvard.edu.

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