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Zebrafish as a model to study peripheral thyroid hormone metabolism in vertebrate development

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ABSTRACT

To unravel the role of thyroid hormones (THs) in vertebrate development it is important to have suitable animal models to study the mechanisms regulating TH availability and activity. Zebrafish (*Danio rerio*), with its rapidly and externally developing transparent embryo has been a widely used model in developmental biology for some time. To date many of the components of the zebrafish thyroid axis have been identified, including the TH transporters MCT8, MCT10 and OATP1C1, the deiodinases D1, D2 and D3, and the receptors TRα and TRβ. Their structure and function closely resemble those of higher vertebrates. Interestingly, due to a whole genome duplication in the early evolution of ray-finned fishes, zebrafish possess two genes for D3 (*dio3* and *dio3a*) and for TRα (*thraa* and *thrab*). Transcripts of all identified genes are present during embryonic development and several of them show dynamic spatio-temporal distribution patterns. Transient morpholino-knockdown of D2, D3 or MCT8 expression clearly disturbs embryonic development, confirming the importance of each of these regulators during early life stages. The recently available tools for targeted stable gene knockout will further increase the value of zebrafish to study the role of peripheral TH metabolism in pre- and post-hatch/post-natal vertebrate development.

1. Introduction

Thyroid hormones (THs) are active in nearly every cell in the vertebrate body. They are crucial for the regulation of development, growth, and metabolism in general. Despite some anatomical differences, the thyroid system is highly conserved among vertebrate species. As in other vertebrates, the functional unit of the zebrafish thyroid is the follicle, composed of endoderm-derived thyrocytes. However, as in most teleosts, the thyroid follicles are dispersed in the pharyngeal region without forming a compact gland (Wendl et al., 2002). The main TH secreted by the thyroid follicles is 3,5,3′,5′-tetraiodothyronine or thyroxine (T_4), while the majority of the most active form of TH, 3,5,3′-triiodothyronine (T_3), is generated in peripheral tissues by deiodination of T_4. At the cellular level, THs exert their functions by binding to nuclear thyroid hormone receptors (TRs) and thus regulating the expression of target genes.

With the increasing number of reports on thyroid-disrupting chemicals in the environment, zebrafish embryos and larvae have recently become very popular as they provide a suitable vertebrate model to screen for thyroid-disrupting chemical pollutants, unraveling their mode of action, and evaluating their risk to animals and humans (Chen et al., 2012; Liu et al., 2011; Raldua and Babin, 2009, 2012; Shi et al., 2009; Thienpont et al., 2011; Yu et al., 2010, 2011). The small size, the ease of maintenance and culture, the rapid embryonic development, and the optical transparency of developing embryos constitute just a few factors making zebrafish an attractive model organism to work with. The widely used morpholino anti-sense knockdown technique and the more recently emerging knockout technology using zinc-finger or transcription activator-like effector nucleases, also make it a favorite model organism to reveal gene functions. Many of the components of the zebrafish thyroid axis have been identified to date, and their structure and function closely resemble those of higher vertebrates. For all these reasons, the zebrafish is a suitable model to gain insight in peripheral TH metabolism during vertebrate development. In this mini-review we have summarized the current understanding of peripheral TH metabolism in zebrafish based on

Abbreviations: D1, type 1 deiodinase; D2, type 2 deiodinase; D3, type 3 deiodinase; DTT, dithiothreitol; dpf, days post fertilization; hpf, hours post fertilization; IRD, inner ring deiodination; ISH, *in situ* hybridization; KO, knockout; MCT, monocarboxylate transporter; MO, morpholino oligonucleotide; ORD, outer ring deiodination; qRT-PCR, quantitative reverse transcription polymerase chain reaction; OATP, organic anion transporting polypeptide; PTU, 6-*n*-propyl-2-thiouracil; T_3, 3,5,3′-triiodothyronine; T_4, 3,5,3′,5′-tetraiodothyronine or thyroxine; TALEN, transcription activator-like effector nuclease; TH, thyroid hormone; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; ZFN, zinc-finger nuclease.

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literature data complemented with some recent findings of our own research group.

2. Development of the zebrafish thyroid gland and pituitary

A schematic representation of the main events in the development of the thyroid gland and the changes in T_4 and T_3 concentrations during zebrafish development is shown in Fig. 1.

The thyroid gland originates from the endoderm layer during vertebrate embryonic development. Zebrafish thyroid precursor cell markers *nkx2.1a*, *hhex* and *pax2a* can be detected as soon as 24 h post fertilization (hpf) (Rohr and Concha, 2000; Wendl et al., 2002). At about 32 hpf the thyroid primordium evaginates from the pharyngeal epithelium (Opitz et al., 2012), and two hours later *tg* (thyroglobulin) mRNA expression is first observed (Fig. 1) (Alt et al., 2006; Opitz et al., 2011). Between 40 and 42 hpf mRNA expression of *slc5a5* (the sodium/iodide symporter NIS) and *tpo* (thyroid peroxidase) becomes detectable in the thyroid primordium (Fig. 1) (Alt et al., 2006; Opitz et al., 2011). Thyroglobulin immunostaining indicates that the first thyroid follicle is formed at 55 hpf (Alt et al., 2006), and the gland starts to produce T_4 at 72 hpf (Fig. 1) (Elsalini et al., 2003). Correct thyroid gland development and differentiation in zebrafish is orchestrated among other things by the Nodal and Notch signaling pathways (Porazzi et al., 2009, 2012).

Although Alt et al. (2006) reported that formation, growth and differentiation of thyroid follicles appears to be independent of thyroid-stimulating hormone (TSH) in zebrafish, a more recent study of Opitz et al. (2011) demonstrates the requirement of zebrafish TSH receptor function for thyroid gland differentiation. TSH produced by the pituitary stimulates the thyroid to produce THs. During zebrafish development, adenohypophysis precursor cells become morphologically distinct at 18–20 hpf (Chapman et al., 2005). By 24 hpf these cells are identifiable as pituitary anlage, and are situated rostroventral to the developing hypothalamus and caudoventral to the stomodeal cells (Chapman et al., 2005). By 36 hpf concurrent mRNA expression of both TSH subunits occurs in the pituitary anlage (Nica et al., 2004; Opitz et al., 2011), and mRNA expression of *tshr* (TSH receptor) in the thyroid primordium starts at 40 hpf (Fig. 1) (Opitz et al., 2011) so by then

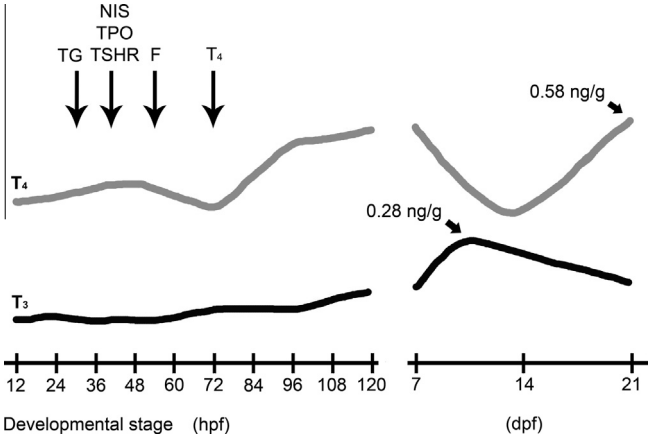

Fig. 1. Schematic representation of the ontogeny of thyroid gland development and hormone secretion in zebrafish. Onset of mRNA expression of thyroglobulin (TG), sodium/iodide symporter (NIS), thyroid peroxidase (TPO), thyroid-stimulating hormone receptor (TSHR) and detection of the first differentiated follicle (F) and embryonic T_4 production (T_4) are indicated with arrows. Peak values of T_4 and T_3 are also specified. Adapted from Chang et al. (2012) including information from (Alt et al., 2006; Elsalini et al., 2003; Opitz et al., 2011)

the further development and functioning of the thyroid gland can be controlled by the pituitary.

3. TH levels during zebrafish development

THs of maternal origin are present in high quantities in fish eggs (Power et al., 2001). Levels measured in zebrafish eggs vary between 0.1–0.2 ng/g for T_3 and 0.3–2.7 ng/g for T_4 (Chang et al., 2012; Walpita et al., 2007). The high ratio of T_4:T_3 found in zebrafish eggs is in accordance with studies in eggs from other fresh water fish (Power et al., 2001). The whole-body content of T_4 and T_3 remains relatively stable during the pre-hatch period (0–3 days post fertilization, dpf) (Fig. 1) (Chang et al., 2012; Walpita et al., 2007). However, at these early stages whole body content is largely reflecting the maternal TH stock present in the yolk sac. There is a real need for more sensitive methods allowing measurements in embryonic tissues alone. This would fill a major gap in our present knowledge and probably reveal a far more dynamic pattern.

After hatching and activation of the larval thyroid gland, a rapid rise in whole-body T_4 and T_3 content appears at 5 dpf (Fig. 1) (Chang et al., 2012). This can be associated with the importance of THs for the embryonic to larval transitory phase, when major morphological changes take place, such as swim bladder inflation, structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk-sac (Kimmel et al., 1995; Liu and Chan, 2002). Whole-body T_3 content reaches a peak at 10 dpf, and T_4 content again rises to high levels at 21 dpf after an initial decline between 7 and 14 dpf (Fig. 1) (Chang et al., 2012). This rise in T_4 content can most likely be linked to the TH-dependency of the larval to juvenile transition (Brown, 1997; Chang et al., 2012). Subsequently, TH levels gradually decline to a lower plateau in later development (Chang et al., 2012).

4. Expression and distribution of TH transporters during zebrafish development

A key requirement for TH activity is the efficient transport of THs across the cell membrane. Despite the lipophilic nature of T_3 and T_4, this transport does not happen by passive diffusion but by an ATP-dependent carrier-mediated transport. In recent years, several TH membrane transporters have been identified in mammals, including monocarboxylate transporter 8 (MCT8), MCT10 and organic anion transporting polypeptide 1C1 (OATP1C1). All of them are members of the solute carrier superfamily of membrane transport proteins. Arjona et al. (2011) recently provided the first evidence for TH transporters in fish. They cloned the coding sequence of the zebrafish solute carrier *slc16a2* (or *mct8*) gene and established the role of MCT8 as a TH transport protein. The amino acid sequence of zebrafish MCT8 shares a high sequence homology with MCT8 of human, mouse and rat.

Mct8 mRNA is expressed during embryonic development, from the earliest time point studied (3 hpf), and the highest embryonic expression occurs at 48 hpf. In adult zebrafish, *mct8* mRNA is highly expressed in brain, gills, pancreas, liver, pituitary, heart, kidney and gut, a tissue distribution that is similar to that in rat (Arjona et al., 2011; Friesema et al., 2003). Recently, an elaborate study of the spatial and temporal expression patterns of zebrafish *mct8* mRNA was carried out by means of whole mount *in situ* hybridization (ISH) and the use of an *mct8* promotor-driven transgenic zebrafish line (Vatine et al., 2013). At 10 hpf *mct8* mRNA is ubiquitously expressed, and at 24–48 hpf expression occurs mainly in the central nervous system (in the whole brain and along the spinal cord) and the notochord. Vatine et al. (2013) also localized *slco1c1* (or *oatp1c1*) and *mct10* mRNA expression at 48 hpf in vascular structures and in the liver and trigeminal ganglia

respectively, but experimental evidence for TH transport by the proteins encoded by zebrafish *oatp1c1* and *mct10* has not yet been provided.

5. Expression and distribution of deiodinases during zebrafish development

Intracellular concentrations of active and inactive THs are tightly regulated, in a tissue-specific manner, by peripheral deiodination. TH activation occurs when iodine is removed from the phenolic ring of T_4 by means of outer ring deiodination (ORD) to form T_3. Inactivation of TH takes place when iodine is removed from the inner tyrosyl ring of T_4 or T_3 (inner ring deiodination or IRD) to form the biologically inactive 3,3′,5′-triiodothyronine (reverse T_3) or 3,3′-diiodothyronine (T_2) respectively (Fig. 2). All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain the rare amino acid selenocysteine in their catalytic centre. D1 is a dual-function deiodinase as it is capable of catalyzing both activation and inactivation of THs, whereas D2 only catalyzes activation and D3 only catalyzes inactivation. In fish, three types of deiodinases are present – encoded by *dio1*, *dio2* and *dio3* – each with a structure very similar to those described in other vertebrates (Orozco and Valverde, 2005). Like in other vertebrates zebrafish *dio3* contains no intron interrupting the open reading frame (Bouzaffour et al., 2010), in contrast to *dio1* and *dio2*. The function and characteristics of the different types of deiodinases are also largely conserved, although there are a few notable exceptions. One is the resistance of teleostean (and amphibian) D1s to inhibition by 6-*n*-propyl-2-thiouracil (PTU), whereas a high PTU-sensitivity is one of the typical characteristics defined for D1 in higher vertebrates (Orozco et al., 2003; Sanders et al., 1997). Another exception concerns the effect of the reducing thiol cosubstrate dithiothreitol (DTT) on teleostean ORD reactions *in vitro*. The stimulating capacity of DTT seems to vary between teleost species and even between tissues within the same species (Klaren et al., 2012; Mol et al., 1997, 1998; Orozco et al., 1997, 2000). Even inhibitory responses to DTT have been reported in some teleost species (Arjona et al., 2008; Klaren et al., 2005; Orozco et al., 2000). For zebrafish, the effect of DTT on ORD activity remains to be elucidated.

Using quantitative reverse transcription polymerase chain reaction (qRT-PCR), *dio1* and *dio2* mRNA was detected from the earliest stages studied (8 hpf). *Dio1* expression shows a threefold increase from 8 to 75 hpf, whereas *dio2* expression is maintained at low and stable levels during embryonic development. *Dio2* expression however increases dramatically immediately after hatching, as shown by a fivefold increase at 75 hpf compared to embryonic levels (Walpita et al., 2007).

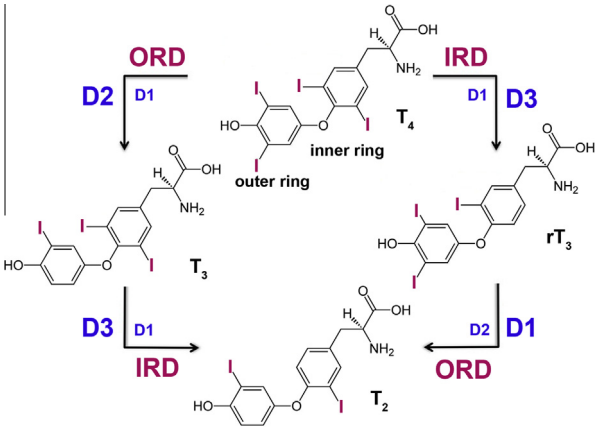

Fig. 2. Major pathways of TH deiodination in vertebrates. D1–D3: deiodinase type 1–3, IRD: inner ring deiodination, ORD: outer ring deiodination.

Table 1
Ontogeny of the expression of deiodinases during zebrafish development as shown by whole mount *in situ* hybridization.

Stage	mRNA		
	dio1	*dio2*	*dio3*
3 hpf (1 k-cell)	**Whole body (1)**	**Whole body (1)**	**- (1)**
5,3 hpf (50% Epiboly)	**Whole body (1)**	**Whole body (1)**	**- (1)**
12 hpf (6 Somite)	**Head/brain (1)**	**Head/brain (1)**	**Kidney (1)**
24 hpf	Head/brain (3)	**Adenohypophysis (2)** **Retina (2,4)** Head/brain (1)	**Kidney (1)** Head/brain (1)
48 hpf	**Liver (3)** Head/brain (3)	**Adenohypophysis (2)** **Retina (2,4)** **Spinal cord (1)** Head/brain (1,4)	**Kidney (3)**
72 hpf	**Liver (2)** **Kidney (2)** **Intestine (2)** Head/brain (1)	**Adenohypophysis (2,4)** **Retina (4)** Head/brain (1)	?
96 hpf	**Liver (1,2)** **Kidney (2)** **Intestine (2)** **Swim bladder (1)**	**Adenohypophysis (2,4)** Swim bladder (1)	?
120 hpf	**Liver (2)** **Kidney (2)** **Intestine (2)**	**Adenohypophysis (2,4)** **Swim bladder (2)**	?

(1) Dong et al. (2013).
(2) Thisse et al. (2003).
(3) Vatine et al. (2013).
(4) Own unpublished results.
Regular font indicates weak expression, bold font indicates moderate to strong expression.
? : not mentioned, - : no signal.

A summary of the tissue specific mRNA localization and intensity of the three deiodinases, based on the information given below, is shown in Table 1. Thisse et al. (2003) were the first to show *dio2* mRNA expression in zebrafish embryos and expression was localized primarily in the adenohypophysis, retina and intestinal bulb from 24 to 48 hpf. At 5 dpf they observed *dio2* expression in the adenohypophysis and the swim bladder. Vatine et al. (2013) have recently reported *dio2* mRNA expression at 48 hpf in a small region in the head, which they concluded to be the thyroid primordium. Our research group has studied *dio2* mRNA expression in the head region in more detail by means of whole mount ISH followed by paraffin sectioning, and we could clearly localize the *dio2* signal at the dorsal side of the pharynx (Fig. 3A–B, own unpublished results), while the thyroid primordium is located in the subpharyngeal mesenchyme (Alt et al., 2006; Opitz et al., 2012). Furthermore, we also found *dio2* mRNA expression in the adenohypophysis of 4- and 5-day old larvae by means of ISH on cryostat sections (Fig. 3C–D, own unpublished results). Therefore our findings confirm *dio2* expression in the adenohypophysis as suggested before by Thisse et al. (2003). Moreover, in a very recent study of Dong et al. (2013) *dio2* expression has also been localized in the adenohypophysis at 48 hpf. Dong et al. (2013) further demonstrated ubiquitous *dio1* and *dio2* mRNA expression in all cells from the start of development (2-cell stage) until 3 hpf. These transcripts are exclusively of maternal origin, because zygotic mRNA expression does not start until 3 hpf (midblastula transition). *Dio1* and *dio2* expression remained co-localized for the first day of development with expression mostly concentrated in the rostral

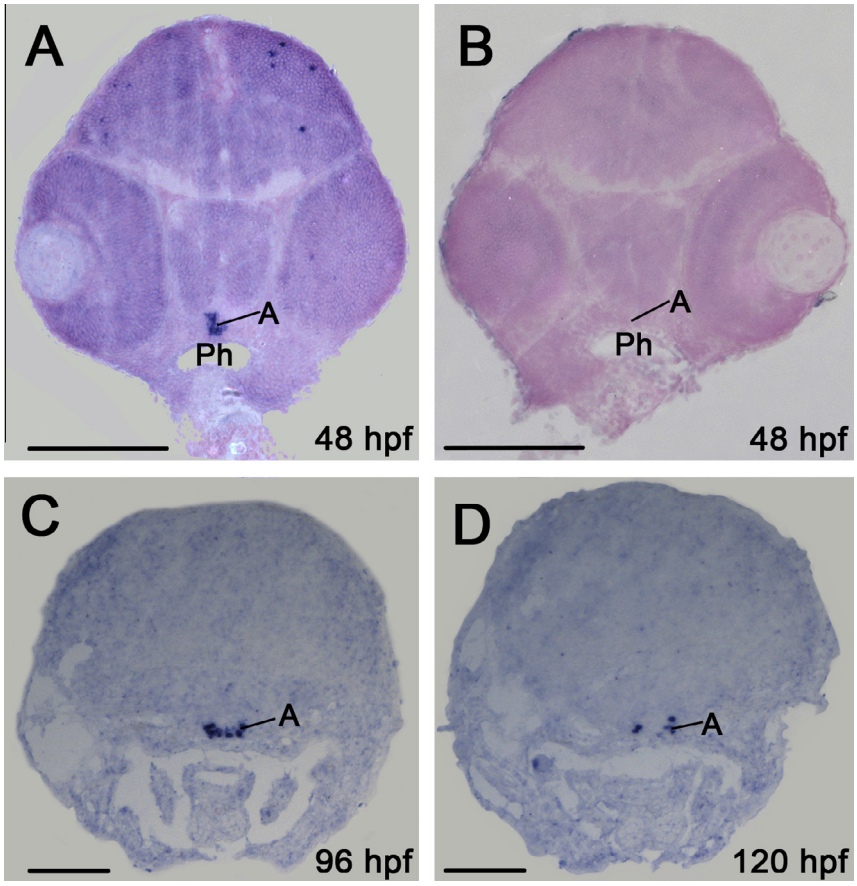

Fig. 3. Expression pattern of *dio2* in developing zebrafish. A–B. Transverse paraffin sections through the head of 48 hpf zebrafish embryos after whole mount ISH with an antisense (A) or sense (B) probe for *dio2*. C–D. ISH for *dio2* on transverse cryostat sections through the head of 96 hpf (C) and 120 hpf (D) zebrafish larvae. Scale bars = 100 μm. A: adenohypophysis, Ph: pharynx.

and caudal regions of the embryo between 12 and 18 hpf and in the periventricular zone of the brain at 22 and 24 hpf (Dong et al., 2013). After 24 hpf the expression pattern of *dio1* mRNA starts to differ from that of *dio2*. Weak *dio1* mRNA expression is found in the head at 48 and 72 hpf (Dong et al., 2013; Vatine et al., 2013), whereas strong expression is found in the liver (48–96 hpf) and in the intestine and kidney (72–96 hpf) (Dong et al., 2013; Thisse et al., 2003; Vatine et al., 2013). Up to 10 dpf strong *dio1* expression has been detected in the liver and interrenal tissue (Dong et al., 2013).

Although D1 is capable of both outer and inner ring deiodination, the prime inactivating deiodinase in fish as well as in other vertebrates is D3. In vertebrates in general, D3 is highly expressed during embryonic/fetal development. In zebrafish embryos, maternal *dio3* mRNA is present at 1 hpf at relatively high levels, that quickly drop towards 24 hpf. A low and stable *dio3* mRNA expression in whole embryos has been recorded from 24 to 72 hpf (Walpita, 2008). Recently, *dio3* mRNA has been localized in the pronephros of zebrafish embryos at 12 hpf, 24 hpf and 48 hpf (Dong et al., 2013; Vatine et al., 2013) as well as a weak expression in the mid-brain region at 24 hpf. D3 is also present in adult zebrafish and *dio3* mRNA and D3 enzyme activity are highly induced in the fin after partial amputation (Bouzaffour et al., 2010). In mammals, D3 is also upregulated during regeneration processes where it prevents local TH action and thus promotes an increase in cellular proliferation (Kester et al., 2009).

Due to a whole genome duplication in the early evolution of ray-finned fishes (Taylor et al., 2003), some teleosts were found to have two *dio3* paralogues (Itoh et al., 2010; Mariotti et al., 2012). Since April 2012, a sequence for a second zebrafish *dio3* gene, named *dio3a*, is available in GenBank (Accession No. NM_001256003). By means of qRT-PCR we have recently shown that both genes are expressed in embryonic as well as adult zebrafish (own unpublished results). However it remains to be investigated if these mRNAs are both translated to functional proteins.

So far all data on deiodinase expression in zebrafish development is based on mRNA expression profiles. Due to the lack of appropriate antibodies it is currently unknown whether these mRNAs are translated to proteins. In addition, significant post-translational regulation of enzyme activities occurs in the case of deiodinases (e.g. substrate-induced down-regulation or ubiquitination and subsequent proteasomal degradation of D2 (Bianco and Larsen, 2005), and endocytosis and recycling of D3 (Baqui et al., 2003)). To the best of our knowledge there is no information available yet on deiodinase activities in zebrafish embryos.

6. Expression and distribution of TRs during zebrafish development

At the cellular level, TH signaling results mainly from the interaction of T_3 and to a smaller extent T_4 with the TRs. In concert with other transcription factors, TRs can stimulate or repress the expression of target genes. In the absence of hormone, TRs interact with corepressors that are released upon hormone binding and replaced by coactivators.

Most vertebrates only have two TR genes, encoding thyroid hormone receptor alpha (TRα) and thyroid hormone receptor beta (TRβ), respectively. Originally only TRα1 and TRβ1 transcripts were identified for zebrafish (Essner et al., 1997; Liu et al., 2000).

However, due to the ancestral teleost gene duplication, several fish species have two TRα encoding genes, and both genes are expressed in zebrafish (Bertrand et al., 2007; Takayama et al., 2008). The originally identified zebrafish gene for TRα has therefore been renamed *thraa*, while the second one is called *thrab*. The *thraa* gene gives rise to at least two proteins: TRαA1 and TRαA1–2. The TRαA1 isoform differs from other TRs by containing an additional C-terminal 'F-domain' extension that reduces the transcriptional activity of the receptor by altering its affinity for the zebrafish coactivator NCoA2 (Takayama et al., 2008). For the TRβ receptor, two isoforms have been described: TRβ1 and TRβ2 (Marchand et al., 2001).

The first TR expression studies have demonstrated maternal *thraa* and *thrb* transcripts in the zebrafish zygote at the 1-cell stage (Essner et al., 1997; Liu et al., 2000). *Thraa* transcripts remain detectable until the 8-cell stage and expression levels are higher than those of *thrb* transcripts, which seem to be degraded by the 2-cell stage. Zygotic expression of *thraa* and *thrb* mRNA is already apparent at the 8- and 16-cell stage respectively, which is well before the midblastula transition in zebrafish, and the increase in *thraa* transcripts preceeds that of *thrb* (Liu et al., 2000). Our research group measured relatively high *thraa* mRNA levels at 8 hpf followed by low levels up till hatching (Walpita et al., 2007). This is in line with the results of Liu and Chan (2002) showing low and relatively constant *thraa* mRNA levels from 1 to 3 dpf. After hatching, *thraa* levels show a peak at 5 dpf (Liu and Chan, 2002). For *thrb* we found more or less stable mRNA levels throughout embryonic development, followed by a rapid increase around hatching (Darras et al., 2011; Walpita, 2008). However, Liu and Chan found the highest increase in embryonic *thrb* expression between 1 and 2 dpf and expression levels remained high until 6 dpf, before declining by 24% on 7 dpf (Liu and Chan, 2002).

Most of the TRα qRT-PCR expression data available to date were obtained using primers based exclusively on the *thraa* sequence. Takayama and colleagues (Takayama et al., 2008) were the first to provide expression data of *thrab* and the first to distinguish between the different *thraa* transcripts. They showed that significant amounts of TRαA1-encoding transcripts are only present in testes and ovaries of adult zebrafish, and in unfertilized eggs and embryos up to 4 hpf. They also showed that, in contrast to the expression of TRαA1-encoding transcripts, the expression level of TRαA1–2-encoding transcripts increases 5-fold and that of *thrab* transcripts increases 28-fold between 1 and 4 dpf. This stage- and tissue-specific expression pattern of the different TRα isoforms suggests specific functions for each isoform. For instance, the high expression of TRαA1 in gonads and in unfertilized eggs (Takayama et al., 2008) may indicate a role of this particular isoform in mediating the effect of THs on gamete differentiation and maturation.

Little is known about the exact localization of TR expression during zebrafish development except for the fact that *thrb* is expressed in the retina from approximately 30 hpf onwards and also in the mid- and hindbrain from approximately 40 hpf onwards (Bertrand et al., 2007; Thisse and Thisse, 2008). This emphasizes the need for further TR localization studies, as tissues with elevated TR expression are probably involved in TH action during development, and may likely serve as powerful tools for further exposing the functions of TRs, deiodinases and TH transporters.

7. Zebrafish as a model for TH-related gene silencing

Since the availability of morpholino oligonucleotides (MOs) zebrafish has been an excellent model for loss-of-function studies. MOs are synthetic oligomers and consist of 25 morpholine bases that can either block proper translation or induce incorrect splicing of the mRNA of interest by complementary base pairing, resulting in a gene-specific transient knockdown (Bedell et al., 2011; Bill et al., 2009). Gene-specific knockout (KO) technology was introduced only recently in zebrafish; first by using engineered zinc-finger nucleases (ZFNs) (Meng et al., 2008) and more recently by employing transcription activator-like effector nucleases (TALENs) (Bedell et al., 2012; Sander et al., 2011). The method of action of the ZFN- and TALEN-technology is basically the same, but their structure slightly differs. ZFNs are DNA-binding proteins consisting of a chimeric fusion between Cys_2His_2 zinc-finger proteins and the nonspecific cleavage domain of *Fok*I endonuclease. TALENs comprise an engineered array of transcription activator-like effector repeats fused to the *Fok*I cleavage domain. Both techniques introduce targeted double-stranded DNA-breaks, which can lead to mutations when repaired by non-homologous end joining, but TALENs seem to be even more specific and efficient than ZFNs (Moore et al., 2012). These innovative and efficient techniques now allow the creation of targeted mutations in TH-related genes, including TH transporters, deiodinases and TRs, to study their specific function during zebrafish development. Interestingly, the Zebrafish Mutation Project aims to create a KO allele in every protein-coding gene in the zebrafish genome, using a combination of whole exome enrichment and Illumina next generation sequencing (http://www.sanger.ac.uk/Projects/D_rerio/zmp/). When a KO allele is successfully generated, fish carrying the allele can be requested from the Zebrafish International Resource Center (ZIRC). To date *thrb* mutants are already available but their phenotype has not yet been analyzed. For *tshr*, *tg*, *slco1c1* and *dio3*, mutant fish will probably be available soon.

Our research group started several years ago to study the function of D1 and D2 during zebrafish embryonic development using MO knockdown. The injection of a D1-targeted MO (D1MO) alone did not have apparent effects on development prior to hatching. In contrast, two different D2MOs each resulted in a clear developmental delay as demonstrated by three different morphological parameters: otic vesicle length, head-to-trunk angle and pigmentation index measured at 31 hpf (Walpita et al., 2009). Interestingly, the combined injection of D1MO and D2MO (each at half the concentration as for the single knockdown) not only provoked a more pronounced developmental delay, but also the occurrence of 20–27% embryos with clearly abnormal morphology (Walpita et al., 2010). This suggests that D2 is the major contributor to TH activation in developing embryos, while D1-mediated activation only seems crucial under depleted thyroid status when D2 activity is greatly reduced. It was indeed shown that relative *dio1* mRNA expression was mildly increased in D2 knockdown embryos (Walpita et al., 2010). Proof that depleted intracellular T_3 availability was the cause of the developmental defects triggered by co-knockdown of D1 and D2, came from the observation that the D1D2-knockdown phenotype could be effectively rescued by addition of T_3, but not T_4, to the embryo medium (Walpita et al., 2010). In comparison, it is interesting to note that although D1 in mammals is considered far more important for the peripheral production of T_3 than in fish, D1KO mice also show no apparent developmental deficits, while D2KO mice display a mild neurological phenotype accompanied by a slight postnatal growth retardation in male pups (Galton et al., 2007; Schneider et al., 2001, 2006). In addition, but in contrast to the results obtained for combined D1D2-knockdown in zebrafish, the phenotype of the D1D2KO mouse is not substantially different from that described for the single D2KO (Galton et al., 2009). One of the major reasons for this mild phenotype in the D1D2KO pups may be that circulating T_3 levels in the mature D1D2KO mice are not decreased while T_4 levels are highly increased. As a result an enhanced TH transfer from the dam to the developing embryo may reduce the impact of deiodinase deficiency. The exclusion of such maternal compensatory mechanisms in the externally developing zebrafish embryos therefore makes

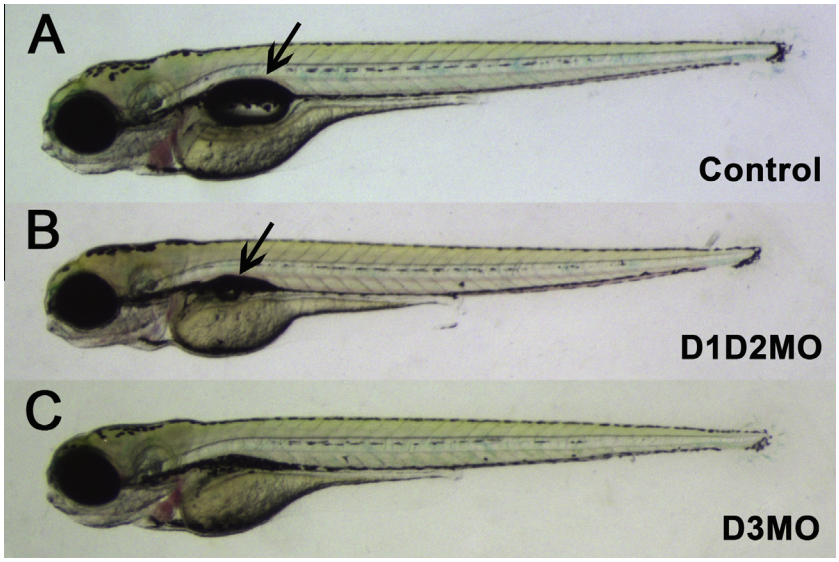

Fig. 4. Knockdown of deiodinases alters swim bladder development in zebrafish larvae. A–C. Lateral views of representative 4 dpf larvae injected with a standard control MO (A) or a D3-targeted MO (D3MO) (C), or injected with a combination of a D1-targeted and a D2-targeted MO (D1D2MO) (B). Arrows indicate the swim bladder in (A) and (B). Abnormal swim bladder development is apparent in (B) and (C).

this model very suitable for the study of the importance of peripheral TH metabolism during embryonic development.

Recently, we have continued our study of combined D1 and D2 knockdown in zebrafish embryos using lower MO concentrations, in order to avoid the occurrence of clearly abnormal embryos while still inducing a delay in embryonic development. When further expanding our research to early larval stages, we observed a clear defect in the development of the swim bladder (Fig. 4B, own unpublished results). This result is in line with the findings of Liu and Chan (2002) that co-treatment with the goitrogen methimazole and the TR antagonist amiodarone, causes defects in swim bladder development in zebrafish larvae. In the same research paper, these authors showed that similar defects in swim bladder development occur if the larvae are treated with excessive amounts of T_3 or T_4. It is indeed known that appropriate levels of TH at the right time are critical for normal vertebrate development, and D3 can prevent exposure of the embryo to excessive THs coming from the yolk. We are presently investigating the function of D3 and D3a during zebrafish development. From the first results we can conclude that several of the observed defects are similar but more pronounced compared to the ones found following D1D2 knockdown, including a defect in swim bladder development (Fig. 4C, own unpublished results). D3a knockdown seems to produce a similar but milder phenotype compared to D3 knockdown, suggesting that D3 and D3a may be at least partially redundant. Further studies are ongoing to define the phenotype of D1D2- and D3 knockdown in more detail and unravel the underlying mechanisms.

Although injection of MOs is a rapid way of blocking gene expression, the knockdown is only transient. Therefore the recent ZFN and TALEN technology, allowing the creation of stable KOs, provides the possibility to study the effect of blocking deiodinase expression also at later stages of development. For the study of the function of TH membrane transporters and TRs, zebrafish KOs would also come in handy. MCT8-deficient mice were generated and they exhibit impaired TH levels. However, in contrast to human patients with MCT8 mutations, they lack clear neurological defects (Dempsey et al., 1993; Trajkovic et al., 2007). Interestingly, a recent study of MCT8 knockdown in zebrafish using MOs revealed that in this species MCT8 is strictly required for development of the nervous system (Vatine et al., 2013). The availability of a MCT8KO zebrafish would allow studying MCT8 function at later developmental stages. To date, the zebrafish TR genes have escaped notice in the study of gene function using MO knockdown, and other than the recent TRβKO, no mutants are available. Detailed studies of zebrafish TR-mutant phenotypes are nevertheless essential to be able to link the different TR isoforms to specific processes in embryonic development.

8. Conclusion

The key factors involved in vertebrate peripheral TH metabolism – TH transporters, deiodinases and receptors – are all present in zebrafish from the start of embryonic development and great similarities exist in their structure, function and distribution compared to higher vertebrates. An important challenge for the future, however, is to extend the current information at the mRNA level to data on the ontogenetic pattern of functional proteins. Loss-of-function studies with MOs have already provided some interesting clues on the role of TH transporters and deiodinases in embryonic development, although a more detailed analysis of the resulting phenotypes is needed. Performing MO knockdown in specific transgenic reporter lines will certainly allow more detailed morphological descriptions and more powerful conclusions concerning affected cell types or organs in the near future. Finally, new molecular tools are available today making stable targeted gene knockout in zebrafish relatively easy. This will push the model rapidly further on the road to unravel the importance of peripheral TH metabolism in vertebrate development and later life.

References

Alt, B., Reibe, S., Feitosa, N.M., Elsalini, O.A., Wendl, T., Rohr, K.B., 2006. Analysis of origin and growth of the thyroid gland in zebrafish. Dev. Dyn. 235, 1872–1883.
Arjona, F.J., de Vrieze, E., Visser, T.J., Flik, G., Klaren, P.H., 2011. Identification and Functional Characterization of Zebrafish Solute Carrier Slc16a2 (Mct8) as a Thyroid Hormone Membrane Transporter. Endocrinology 152, 5065–5073.
Arjona, F.J., Vargas-Chacoff, L., Martin del Rio, M.P., Flik, G., Mancera, J.M., Klaren, P.H., 2008. The involvement of thyroid hormones and cortisol in the osmotic acclimation of Solea senegalensis. Gen. Comp. Endocrinol. 155, 796–803.
Baqui, M., Botero, D., Gereben, B., Curcio, C., Harney, J.W., Salvatore, D., et al., 2003. Human type 3 iodothyronine selenodeiodinase is located in the plasma

membrane and undergoes rapid internalization to endosomes. J. Biol. Chem. 278, 1206–1211.
Bedell, V.M., Wang, Y., Campbell, J.M., Poshusta, T.L., Starker, C.G., Krug 2nd, R.G., et al., 2012. In vivo genome editing using a high-efficiency TALEN system. Nature 491, 114–118.
Bedell, V.M., Westcot, S.E., Ekker, S.C., 2011. Lessons from morpholino-based screening in zebrafish. Brief. Funct. Genomics. 10, 181–188.
Bertrand, S., Thisse, B., Tavares, R., Sachs, L., Chaumot, A., Bardet, P.L., et al., 2007. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. PLoS Genet. 3, e188.
Bianco, A.C., Larsen, P.R., 2005. Cellular and structural biology of the deiodinases. Thyroid 15, 777–786.
Bill, B.R., Petzold, A.M., Clark, K.J., Schimmenti, L.A., Ekker, S.C., 2009. A primer for morpholino use in zebrafish. Zebrafish 6, 69–77.
Bouzaffour, M., Rampon, C., Ramauge, M., Courtin, F., Vriz, S., 2010. Implication of type 3 deiodinase induction in zebrafish fin regeneration. Gen. Comp. Endocrinol. 168, 88–94.
Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. Proc. Natl. Acad. Sci. USA 94, 13011–13016.
Chang, J., Wang, M., Gui, W., Zhao, Y., Yu, L., Zhu, G., 2012. Changes in thyroid hormone levels during zebrafish development. Zoolog. Sci. 29, 181–184.
Chapman, S.C., Sawitzke, A.L., Campbell, D.S., Schoenwolf, G.C., 2005. A three-dimensional atlas of pituitary gland development in the zebrafish. J. Comp. Neurol. 487, 428–440.
Chen, Q., Yu, L., Yang, L., Zhou, B., 2012. Bioconcentration and metabolism of decabromodiphenyl ether (BDE-209) result in thyroid endocrine disruption in zebrafish larvae. Aquat. Toxicol. 110–111, 141–148.
Darras, V.M., Van Herck, S.L., Heijlen, M., De Groef, B., 2011. Thyroid hormone receptors in two model species for vertebrate embryonic development: chicken and zebrafish. J. Thyroid. Res. 2011, 402320.
Dempsey, M.A., Dumitrescu, A.M., Refetoff, S., 1993. In: Pagon, R.A., Bird, T.D., Dolan, C.R., Stephens, K., Adam, M.P. (Eds.), MCT8 (SLC16A2)-Specific Thyroid Hormone Cell Transporter Deficiency. GeneReviews, Seattle (WA).
Dong, W., Macaulay, L.J., Kwok, K.W.H., Hinton, D.E., Stapleton, H.M., 2013. Using whole mount *in situ* hybridization to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish: a tool for examining OH-BDE toxicity to early life stages. Aquat. Toxicol. 132–133, 190–199.
Elsalini, O.A., von Gartzen, J., Cramer, M., Rohr, K.B., 2003. Zebrafish hhex, nk2.1a, and pax2.1 regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors. Dev. Biol. 263, 67–80.
Essner, J.J., Breuer, J.J., Essner, R.D., Fahrenkrug, S.C., Hackett Jr., P.B., 1997. The zebrafish thyroid hormone receptor alpha 1 is expressed during early embryogenesis and can function in transcriptional repression. Differentiation 62, 107–117.
Friesema, E.C., Ganguly, S., Abdalla, A., Manning Fox, J.E., Halestrap, A.P., Visser, T.J., 2003. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. J. Biol. Chem. 278, 40128–40135.
Galton, V.A., Schneider, M.J., Clark, A.S., St Germain, D.L., 2009. Life without thyroxine to 3,5,3′-triiodothyronine conversion: studies in mice devoid of the 5′-deiodinases. Endocrinology 150, 2957–2963.
Galton, V.A., Wood, E.T., St Germain, E.A., Withrow, C.A., Aldrich, G., St Germain, G.M., et al., 2007. Thyroid hormone homeostasis and action in the type 2 deiodinase-deficient rodent brain during development. Endocrinology 148, 3080–3088.
Itoh, K., Watanabe, K., Wu, X., Suzuki, T., 2010. Three members of the iodothyronine deiodinase family, dio1, dio2 and dio3, are expressed in spatially and temporally specific patterns during metamorphosis of the flounder, Paralichthys olivaceus. Zoolog. Sci. 27, 574–580.
Kester, M.H., Toussaint, M.J., Punt, C.A., Matondo, R., Aarnio, A.M., Darras, V.M., et al., 2009. Large induction of type III deiodinase expression after partial hepatectomy in the regenerating mouse and rat liver. Endocrinology 150, 540–545.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.
Klaren, P.H., Geven, E.J., Nagelkerke, A., Flik, G., 2012. Kinetics and thiol requirements of iodothyronine 5'-deiodination are tissue-specific in common carp (Cyprinus carpio L.). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 161, 275–282.
Klaren, P.H., Haasdijk, R., Metz, J.R., Nitsch, L.M., Darras, V.M., Van der Geyten, S., et al., 2005. Characterization of an iodothyronine 5′-deiodinase in gilthead seabream (Sparus auratus) that is inhibited by dithiothreitol. Endocrinology 146, 5621–5630.
Liu, Y., Wang, J., Fang, X., Zhang, H., Dai, J., 2011. The thyroid-disrupting effects of long-term perfluorononanoate exposure on zebrafish (Danio rerio). Ecotoxicology 20, 47–55.
Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation 70, 36–45.
Liu, Y.W., Lo, L.J., Chan, W.K., 2000. Temporal expression and T3 induction of thyroid hormone receptors alpha1 and beta1 during early embryonic and larval development in zebrafish, Danio rerio. Mol. Cell. Endocrinol. 159, 187–195.
Marchand, O., Safi, R., Escriva, H., Van Rompaey, E., Prunet, P., Laudet, V., 2001. Molecular cloning and characterization of thyroid hormone receptors in teleost fish. J. Mol. Endocrinol. 26, 51–65.
Mariotti, M., Ridge, P.G., Zhang, Y., Lobanov, A.V., Pringle, T.H., Guigo, R., et al., 2012. Composition and evolution of the vertebrate and mammalian selenoproteomes. PLoS ONE 7, e33066.
Meng, X., Noyes, M.B., Zhu, L.J., Lawson, N.D., Wolfe, S.A., 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat. Biotechnol. 26, 695–701.
Mol, K.A., Van Der Geyten, S., Burel, C., Kuhn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiol. Biochem. 18, 253–266.
Mol, K.A., Van Der Geyten, S., Darras, V.M., Visser, T.J., Kuhn, E.R., 1997. Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, Oreochromis aureus. Endocrinology 138, 1787–1793.
Moore, F.E., Reyon, D., Sander, J.D., Martinez, S.A., Blackburn, J.S., Khayter, C., et al., 2012. Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). PLoS ONE 7, e37877.
Nica, G., Herzog, W., Sonntag, C., Hammerschmidt, M., 2004. Zebrafish pit1 mutants lack three pituitary cell types and develop severe dwarfism. Mol. Endocrinol. 18, 1196–1209.
Opitz, R., Maquet, E., Huisken, J., Antonica, F., Trubiroha, A., Pottier, G., et al., 2012. Transgenic zebrafish illuminate the dynamics of thyroid morphogenesis and its relationship to cardiovascular development. Dev. Biol. 372, 203–216.
Opitz, R., Maquet, E., Zoenen, M., Dadhich, R., Costagliola, S., 2011. TSH receptor function is required for normal thyroid differentiation in zebrafish. Mol. Endocrinol. 25, 1579–1599.
Orozco, A., Linser, P., Valverde, C., 2000. Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish Fundulus heteroclitus. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 126, 283–290.
Orozco, A., Silva, J.E., Valverde, R.C., 1997. Rainbow trout liver expresses two iodothyronine phenolic ring deiodinase pathways with the characteristics of mammalian types I and II 5'-deiodinases. Endocrinology 138, 254–258.
Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. Thyroid 15, 799–813.
Orozco, A., Villalobos, P., Jeziorski, M.C., Valverde, R.C., 2003. The liver of Fundulus heteroclitus expresses deiodinase type 1 mRNA. Gen. Comp. Endocrinol. 130, 84–91.
Porazzi, P., Calebiro, D., Benato, F., Tiso, N., Persani, L., 2009. Thyroid gland development and function in the zebrafish model. Mol. Cell. Endocrinol. 312, 14–23.
Porazzi, P., Marelli, F., Benato, F., de Filippis, T., Calebiro, D., Argenton, F., et al., 2012. Disruptions of global and JAGGED1-mediated notch signaling affect thyroid morphogenesis in the zebrafish. Endocrinology 153, 5645–5658.
Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjornsson, B.T., Einarsdottir, I.E., et al., 2001. Thyroid hormones in growth and development of fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 130, 447–459.
Raldua, D., Babin, P.J., 2009. Simple, rapid zebrafish larva bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function. Environ. Sci. Technol. 43, 6844–6850.
Raldua, D., Thienpont, B., Babin, P.J., 2012. Zebrafish eleutheroembryos as an alternative system for screening chemicals disrupting the mammalian thyroid gland morphogenesis and function. Reprod. Toxicol. 33, 188–197.
Rohr, K.B., Concha, M.L., 2000. Expression of nk2.1a during early development of the thyroid gland in zebrafish. Mech. Dev. 95, 267–270.
Sander, J.D., Cade, L., Khayter, C., Reyon, D., Peterson, R.T., Joung, J.K., et al., 2011. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat. Biotechnol. 29, 697–698.
Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kuhn, E.R., Leonard, J.L., et al., 1997. Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138, 5153–5160.
Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2001. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. Mol. Endocrinol. 15, 2137–2148.
Schneider, M.J., Fiering, S.N., Thai, B., Wu, S.Y., St Germain, E., Parlow, A.F., et al., 2006. Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice. Endocrinology 147, 580–589.
Shi, X., Liu, C., Wu, G., Zhou, B., 2009. Waterborne exposure to PFOS causes disruption of the hypothalamus-pituitary-thyroid axis in zebrafish larvae. Chemosphere 77, 1010–1018.
Takayama, S., Hostick, U., Haendel, M., Eisen, J., Darimont, B., 2008. An F-domain introduced by alternative splicing regulates activity of the zebrafish thyroid hormone receptor alpha. Gen. Comp. Endocrinol. 155, 176–189.
Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res. 13, 382–390.
Thienpont, B., Tingaud-Sequeira, A., Prats, E., Barata, C., Babin, P.J., Raldua, D., 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environ. Sci. Technol. 45, 7525–7532.
Thisse, C., Degrave, A., Kryukov, G.V., Gladyshev, V.N., Obrecht-Pflumio, S., Krol, A., et al., 2003. Spatial and temporal expression patterns of selenoprotein genes during embryogenesis in zebrafish. Gene Expression Patterns 3, 525–532.
Thisse, C., Thisse, B., 2008. Expression from: unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. ZFIN direct data submission. <http://zfin.org>.
Trajkovic, M., Visser, T.J., Mittag, J., Horn, S., Lukas, J., Darras, V.M., et al., 2007. Abnormal thyroid hormone metabolism in mice lacking the monocarboxylate transporter 8. J. Clin. Invest. 117, 627–635.

Vatine, G.D., Zada, D., Lerer-Goldshtein, T., Tovin, A., Malkinson, G., Yaniv, K., et al., 2013. Zebrafish as a model for monocarboxyl transporter 8-deficiency. J. Biol. Chem. 288, 169–180.

Walpita, C.N., 2008. The use of zebrafish (Danio rerio) as a model organism to study the role of thyroid hormones in embryonic development of teleost fish. Ph.D. thesis, K.U. Leuven.

Walpita, C.N., Crawford, A.D., Darras, V.M., 2010. Combined antisense knockdown of type 1 and type 2 iodothyronine deiodinases disrupts embryonic development in zebrafish (Danio rerio). Gen. Comp. Endocrinol. 166, 134–141.

Walpita, C.N., Crawford, A.D., Janssens, E.D., Van der Geyten, S., Darras, V.M., 2009. Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. Endocrinology 150, 530–539.

Walpita, C.N., Van der Geyten, S., Rurangwa, E., Darras, V.M., 2007. The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (Danio rerio) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. Gen. Comp. Endocrinol. 152, 206–214.

Wendl, T., Lun, K., Mione, M., Favor, J., Brand, M., Wilson, S.W., et al., 2002. Pax2.1 is required for the development of thyroid follicles in zebrafish. Development 129, 3751–3760.

Yu, L., Deng, J., Shi, X., Liu, C., Yu, K., Zhou, B., 2010. Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic-pituitary-thyroid axis of zebrafish larvae. Aquat. Toxicol. 97, 226–233.

Yu, L., Lam, J.C., Guo, Y., Wu, R.S., Lam, P.K., Zhou, B., 2011. Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in zebrafish. Environ. Sci. Technol. 45, 10652–10659.