Thyroid hormone receptor beta is critical for intestinal remodeling during *Xenopus tropicalis* metamorphosis

Yuki Shibata, Yuta Tanizaki and Yun-Bo Shi*

**Abstract**

**Background:** Thyroid hormone (T3) is critical for development in all vertebrates. The mechanism underlying T3 effect has been difficult to study due to the uterus-enclosed nature of mammalian embryos. Anuran metamorphosis, which is dependent on T3 but independent of maternal influence, is an excellent model to study the roles of T3 and its receptors (TRs) during vertebrate development. We and others have reported various effects of TR knockout (TRα and TRβ) during *Xenopus tropicalis* development. However, these studies were largely focused on external morphology.

**Results:** We have generated TRβ knockout animals containing an out-frame-mutation of 5 base deletion by using the CRISPR/Cas9 system and observed that TRβ knockout does not affect premetamorphic tadpole development. We have found that the basal expression of direct T3-inducible genes is increased but their upregulation by T3 is reduced in the intestine of premetamorphic homozygous TRβ knockout animals, accompanied by reduced target binding by TR. More importantly, we have observed reduced adult stem cell proliferation and larval epithelial apoptosis in the intestine during T3-induced metamorphosis.

**Conclusions:** Our data suggest that TRβ plays a critical role in intestinal remodeling during metamorphosis.

**Keywords:** Thyroid hormone receptor, *Xenopus tropicalis*, Anuran metamorphosis, Intestinal remodeling, Stem cells, Apoptosis

**Introduction**

Thyroid hormone (T3) is essential for normal development in all vertebrates, particularly the postembryonic developmental period around birth in mammals and metamorphosis in amphibians when plasma T3 levels peak [1–9]. T3 deficiency in human due to iodine deficiency causes congenital hypothyroidism with severe development problems including impaired mental function, retarded physical development and goiter formation [3, 6]. The developmental effects of T3 are believed to be mediated mainly, if not exclusively, by T3 receptors (TRs). When T3 is absent, the unliganded TRs, which can form heterodimers with 9-cis-retinoic acid receptors (RXRs), bind to T3-response elements (TREs) and recruit corepressors to repress T3-inducible genes. After T3 binding to TRs, the liganded TRs recruit coactivators to induce the expression of these T3 target genes [1, 2, 4, 10–23]. A number of mouse models with individual TRα, TRβ, or TRα/TRβ double knockout have been produced and revealed different TR-isoform-dependent, tissue-specific defects in the adult, supporting important roles of the receptors in regulating T3 signaling during mammalian development [24–31].

We have been studying amphibian metamorphosis as a model system for understanding the regulation of postembryonic vertebrate adult organ development by T3. During metamorphosis in anurans such as the

*Correspondence: Shi@helix.nih.gov
Section on Molecular Morphogenesis, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

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pseudo-tetraploid *Xenopus laevis* and its highly related diploid species *Xenopus tropicalis*, T3 induces dramatic tissue modifications in essentially all tissues/ organs [3, 4, 32–36]. Of particular interest among them is intestinal remodeling. In adult vertebrates, the intestinal epithelium undergoes constant self-renewal through stem cell proliferation in the crypt (mammals) or bottom of the epithelial fold (anurans) and the eventual apoptotic death of the differentiated epithelial cells, mainly at the tip of the villus (mammals) or fold (anurans) [37–44]. Interestingly, this self-renewing system is established during intestinal metamorphosis in anurans or the postembryonic period in mammals [45, 46]. In anurans such as *Xenopus tropicalis*, the tadpole intestine is a simple tubular structure with the epithelium surrounded by thin layers of connective tissue and muscles [38, 47]. During metamorphosis, there is a near total degeneration of the larval epithelium, accompanied by concurrent de novo development of the adult epithelial stem cells, which subsequently proliferate and differentiate to from a complex adult epithelial structure resembling that in adult mammals [38, 39, 45, 46, 48–51]. The total dependence of this process on T3 provides a unique opportunity to analyze the roles of unliganded vs. liganded TR during adult organ development, particularly stem cell formation.

Interestingly, when analyzed in the intestine or whole tadpoles, *TRα* is expressed at high levels from premetamorphosis to the end of metamorphosis while *TRβ* expression is low during premetamorphosis but is induced as a direct T3 target gene to peak at the climax of metamorphosis [36, 52–55]. Consistently, gene knockout studies in *Xenopus tropicalis* have shown that *TRα* single knockout accelerates premetamorphic tadpole growth and development (based on hindlimb morphology), likely due to the removal of unliganded *TRα* which leads to depression of T3 target genes [56, 57]. In addition, there is a delay in intestinal remodeling relative to the external morphological changes in the *TRα* single knockout tadpoles [56, 57]. On the other hands, *TRβ* single knockout tadpoles are normal in premetamorphic development based on external morphology but have delayed tail regression during metamorphosis [58, 59]. On the other hand, the hindlimb development and intestinal remodeling have only relatively subtle differences between the *TRβ* knockout and wild type animals during natural metamorphosis [58, 59], a very surprising finding, especially for the intestine given the strong upregulation of *TRβ* during intestinal metamorphosis. Finally, our recent *TR* double knockout study have revealed that removing all TRs leads to precociously adult organ development but prevents/inhibits larval tissue degeneration during metamorphosis with the tadpoles stalling their development at climax of metamorphosis for up to two weeks before death prior to the completion of tail resorption [60]. These findings suggested that TRs play critical, isotype-specific roles during metamorphosis. On the other hand, given the different effects of *TR* knockouts on different organs, it is difficult to interpret whether the delay in intestinal remodeling during natural metamorphosis is due to direct effect on the intestine or indirect consequence of the different effects of the knockouts on different organs, e.g., accelerated metamorphosis of the external organs, which is used to judge developmental stages, may make intestinal remodeling appear delayed relative to external organs even if the knockouts do not affect the intestine directly.

In this study, we have knocked out *Xenopus tropicalis* *TRβ* gene by using CRISPR/Cas9 genome editing technology and studied age- and stage-matched wild type and knockout animals during T3-induced metamorphosis to avoid the potential artifact due to comparing animals of different ages as during natural metamorphosis. We observed no development defects due to the knockout from embryogenesis to premetamorphosis even though there was derepression of T3 target genes in the *TRβ*−/− premetamorphic tadpoles. Examination of the tadpoles at stage 54 with or without T3 has revealed that *TRβ*−/− tadpoles have reduced responses to exogenous T3 treatment, particularly with regard to T3-induced gill resorption and reshaping of the head to a pointy structure. Importantly, T3-induced intestinal remodeling, including length reduction, adult stem cell development and proliferation, and larval epithelial cell death are all inhibited by the *TRβ* knockout, accompanied by reduction in both target binding by TR and T3-upregulation of target genes in the intestine. Our data suggest that *TRβ* is upregulated by T3 in the intestine and in turn feeds back positively to activate downstream genes in the intestine to promote larval cells apoptosis and adult epithelial stem cell development and proliferation.

**Results and discussion**

**Generation of *TRβ* knockout animals**

We previously reported the adaptation of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome editing technology to knock out *Xenopus tropicalis* *TRβ* in the *TRα* knockout heterozygous animals to generate *TR* double knockout tadpoles [60]. We also used this approach to generate *TRβ* single knockout animals. Briefly, we co-injected Cas9 mRNA and CRISPR-short guide RNA (sgRNA) targeting exon 2 that encodes part of the DNA binding domain of TRβ into one-cell stage of fertilized eggs obtained from a *TRα* heterozygous frog (*TRα*+/−=*TRβ*+/+) and wild type frog (*TRα*+/+*TRβ*+/+) (Fig. 1a). We selected offspring (F0 generation) with wild type *TRα* as genotyped by PCR [61] and containing...
mosaic mutations in TRβ \([TRα^{+/+}/TRβ^{/

mosaic}]\) as determined by sequence analysis [60]. After sexual maturation of F0 animals, we crossed them with wild type animals and obtained TRβ heterozygous knockout animals (F1 generation) \([TRβ^{+/−}]\) with a 5 base out-of-frame mutation (Fig. 1b). We raised some of F1 TRβ heterozygous mutant animals to sexual maturity and generated F2 generation, TRβ homozygous knockout animals \([TRβ^{−/−}]\) by intercrossing F1 heterozygous mutant frogs.

**TRβ knockout does not affect embryogenesis and premetamorphosis despite some derepression of T3 target genes expression in premetamorphic tadpoles**

To analyze the effects of TRβ knockout on Xenopus development, we genotyped F2 siblings from mating two TRβ heterozygous animals, 48 tadpoles at 4 days old (around stages 45/46, the onset of feeding) and 101 tadpoles at developmental stage 54, respectively. Genotyping data revealed a Mendelian distribution of the three expected genotypes at both time points (Fig. 1c). We next recorded their age when individual tadpoles reached stage 54 according to their external morphology and measured the total body length and snout-vent length (Fig. 1d, e). There was no significant difference in the three parameters among the three genotypes and all animals had normal morphology. These results suggest that TRβ knockout had no effect on embryogenesis and premetamorphosis, consistent with the very low levels of TRβ expression during these developmental periods [52, 54, 55].

**T3 direct target genes are derepressed in the intestine of TRβ knockout tadpoles at stage 54, accompanied by reduced TR binding**

To investigate the effects of the knockout on the animal intestine, we analyzed the expression of several well-known direct T3 target genes, TRβ [53], klf9 [62], mmp11 [63], and TH/hzip [64], in the intestine of premetamorphic wild type and TRβ knockout tadpoles. The results showed that the expression of all these genes were increased significantly in TRβ\(^{-/-}\) tadpoles (Fig. 2a) (note that the knockout animals expressed a non-functional mutant \(TRα\) mRNA). To see if there were any changes in TRα expression, we analyzed TRα mRNA level and found a small increase in the intestine of premetamorphic TRβ knockout tadpoles compared to the wild type ones (Fig. 2a). To determine whether the increase in T3 target gene expression was due to partial derepression due to the loss of TRβ in the mutant animals, we analyzed TR binding to the well-characterized TRE in the TRβ gene in the intestine of stage 54 tadpoles by ChIP assay [56, 61]. As shown in Fig. 2b, TR binding to the TRE region was significantly decreased in TRβ knockout animals compared with wild type animals. These results suggest that the reduction of TR binding, with the remaining TR binding due to TRα, likely causes a reduction in the repression by unliganded TR, thus leading to the derepression of T3 target genes in premetamorphic tadpoles. Since TRα mRNA level also rises during intestinal metamorphosis, the increase in TRα mRNA level in the premetamorphic TRβ knockout tadpoles may also be due to this derepression, although it is not yet known if TRα is a directly T3 target gene.

**Premetamorphic TRβ knockout tadpoles have a poor intestinal response to exogenous T3 treatment**

To investigate whether TRβ knockout affects intestinal response to exogenous T3 treatment, wild type and TRβ knockout tadpoles at stage 54 were treated with or without T3 for up to 5 days, a long term T3 treatment that can induce the external morphological change such as the formation of a pointy head, the resorption of the gills, and intestinal length reduction in wild type animals (Fig. 3A). However, TRβ knockout tadpoles showed little change after T3 treatment (Fig. 3A). In addition, there was also a delayed reduction in the intestinal length (Fig. 3B). To examine the cellular changes, we stained the intestinal cross-sections with...
methyl green pyronin Y (MGPY), a mixture of methyl green, which binds to DNA, and pyronin Y, which binds to RNA and thus labels very strongly the proliferating cells but poorly the apoptotic cells [51]. As expected, the intestine at stages 54 of both genotypes were composed of mainly a monolayer of larval epithelial cells surrounded by thin connective tissue and muscles, with only a single epithelial fold, the typhlosole (Fig. 3C a, b). After 3 days T3 treatment, as the larval epithelial cells underwent apoptosis, they became poorly labeled,
Fig. 2 Basal expression of TRα and known T3 target genes in premetamorphic tadpoles is increased in TRβ knockout intestine, accompanied by reduced TR binding to the endogenous TRE region. a TRβ knockout increased TRα and T3 target gene expression in premetamorphic tadpoles. Total RNA of three different tissues, intestine, tail, and hindlimb, of stage 54 tadpoles of the three genotypes, was used for qRT-PCR analysis of the expression of TRα and four well-known T3 direct target genes: TRβ, klf9, mmp11, and TH/bzip. The expression levels were normalized against that of rpl8. Asterisks denote statistically significant differences (P < 0.05). Note that in the knockout tadpoles, TRβ mRNA, which had an out-of-frame mutation, was expressed at a higher level, likely due to partial de-repression caused by the lack of TRβ protein expression, just like the other target genes. b ChIP assay reveals reduced TR binding at the TRE region. The intestine sample obtained from at least five tadpoles of both genotypes, wild type and TRβ knockout, at stage 54 and were homogenized together for ChIP assay with antibodies against TR and ID14 as a negative control. The presence of the TRE region or the exon 5, as a negative control, of TRβ gene was determined by PCR. Asterisks (**) indicates statistically significant differences (P < 0.01)
while clusters of strongly labeled proliferating adult stem cells were found between the connective tissue and dying larval epithelial cells in wild type tadpoles (yellow surrounded Fig. 3C e). The intestine of the TRβ knockout tadpoles after 3 days T3 treatment resembled that in the wild type or TRβ knockout stage 54 tadpoles without T3 treatment (Fig. 3C d, f), indicating a lack of or drastically reduced response of the mutant intestine to exogenous T3. These results suggest that TRβ is required for the intestinal remodeling in response to T3.

**Decreased induction of T3 response gene expression is associated with reduced TR binding in the intestine of TRβ knockout tadpoles after short term T3 treatment**

To determine if the lack of intestinal remodeling in the knockout animals was due to reduced target gene expression, we analyzed the expression of some known T3 response genes during intestinal metamorphosis. As shown in Fig. 4, known T3 upregulated genes, including transcription factors TRα, TRβ, klf9 and TH/bzip; and matrix metalloproteinase (MMP) genes, mmp2, mmp9th, mmp11 and mmp14, all but mmp9th had significantly
reduced induction by T3 in the intestine of TRβ knockout tadpoles at stage 54 compared to the wild type intestine. Analyses of two apoptosis-related genes, caspase 3 (casp3) and caspase 9 (casp9), and cell dedifferentiation-related genes, ror2 and wnt5a [66], found that all but wnt5a had significantly lower expression in the knockout intestine compared to the wild type intestine (Fig. 4). In addition, ChIP assays on the T3-treated animal intestine showed that TRβ knockout tadpole intestine had significantly reduced TR binding to the TRE in TRβ gene,
a well-characterized direct T3 target gene in the intestine (Fig. 5). These results suggest that gene activation by liganded TRβ is important for T3-induced intestinal metamorphosis.

**T3-induced epithelial stem cell formation/proliferation and larval epithelial cell death is inhibited/reduced in homozygous TRβ knockout tadpole intestine**

In addition to the reduction in the length of the intestine, the major changes during intestinal metamorphosis involves the near complete degeneration of the larval epithelial cells and concurrent development of adult epithelial stem cells, following by their proliferation and differentiation to form the adult epithelium [38, 39, 45, 46, 48–51]. Thus, we next investigated cell proliferation by in vivo labeling with 5-ethynyl-2′-deoxyuridine (EdU). Wild type and TRβ knockout tadpoles at stage 54 were treated with T3 for up to 5 days to induce metamorphosis and then injected with 1.25 µl of EdU solution 10 mg/ml into the body of each tadpole [67]. The animals were subsequently sacrificed and sectioned for EdU staining to detect EdU positive cells, and for TUNEL staining to detect apoptotic cells in the intestine. EdU staining clearly detected active cell proliferation in the intestine in both wild type and knockout tadpole intestine (Fig. 6A). In the wild type intestine, the number of EdU positive cells gradually increased in response to T3 treatment and clusters of strongly labeled proliferating stem cells appeared after 3 days T3 treatment (Figs. 6A i, j). However, in TRβ knockout tadpoles, relatively few EdU positive cells were detected with no observable proliferating cell clusters after three days (Fig. 6 s, t). Quantitative measurement of the EdU positive area, normalized against Hoechst positive area, showed that the induction of cell proliferation was reduced in TRβ knockout intestine at all time points between 2–5 days of T3 treatment (Fig. 6B).

![Fig. 5 TRβ knockout reduces TR binding to the TRE region in the tadpole intestine after T3 treatment. Five or more age-matched wild type and TRβ knockout tadpoles at stage 54 were kept in 4-l plastic container and treated with or without 10 nM T3 for 18 h. The chromatin of the intestine was isolated and immunoprecipitated with antibodies against TR and ID14 as a negative control. The immunoprecipitated DNA was analyzed by real-time PCR for the presence of the TRE region or the exon 5, which lacks any TRE, of the TRβ gene. Note that TR binding to the TRE region was significantly reduced in the intestine of TRβ knockout tadpoles. Two asterisks (**) indicate statistically significant differences (P < 0.01)](image)
When apoptosis was analyzed by using TUNEL labeling, we observed the peak level of apoptotic cells after 2 days of T3 treatment in wild type intestine (Fig. 7), as we reported before [68]. In contrast, in TRβ knockout tadpoles, the intestine had reduced number of apoptotic cells after 2 days of T3 treatment compared to the wild type animals (Fig. 7). Similar levels of apoptosis were observed between wild type and mutant intestine after 3 or 5 days of T3 treatment. Thus, TRβ knockout inhibited both adult epithelial stem cell development and/or proliferation and larval epithelial cell death during T3-induced intestinal remodeling, demonstrating a critical role of TRβ in intestinal metamorphosis.

Conclusion

Amphibian metamorphosis mimics postembryonic development in mammals and offers a number of advantages for studying how T3 regulates vertebrate development. Earlier knockout studies, largely based on external morphological criteria, have revealed distinct effects of individual TR knockout in Xenopus tropicalis. TRa knockout does not affect embryogenesis but leads to premature initiation of metamorphosis, i.e., reaching the onset of metamorphosis or stage 54 at younger age compared to wild type siblings, as determined based on hindlimb morphology [56, 57]. In addition, TRa knockout delays the metamorphic progression between stage 54 to stage 58, the early metamorphic climax, again as judged based on limb development. Thus, there is a critical role for TRa in limb development. However, TRa knockout has little effect on tail resorption. TRβ knockout also does not affect embryogenesis. It, on the other hand, has no effect on premetamorphic development or early metamorphosis, suggesting that it does not have a critical role in limb development (Fig. 1) [58, 59]. However, it causes severe delays in tail resorption, particularly notochord resorption [58, 59]. Interestingly, limited studies on the intestine, an internal organ, have revealed relative minor effects of TRa or TRβ knockout during natural metamorphosis, mainly a delay in intestinal remodeling relative to the external morphological changes in the knockout tadpoles [56–59]. This seems to contrast with the strong upregulation of TRβ during intestinal metamorphosis in both Xenopus laevis and Xenopus tropicalis [36, 54, 55], two related anuran species that undergo very similar intestinal remodeling [38, 47]. It is possible that the relatively minor effects observed for the TR knockouts during natural intestinal metamorphosis is in part due to comparing wild type and knockout animals of different ages (in order to match the stage) and/or due to distinct, developmental stage-dependent effects of the TR knockouts on the intestine and external organs that are used to determine the stages of the animals.

By using age- and stage-matched wild type and TRβ knockout tadpoles to study T3-induced metamorphosis, we have demonstrated here clearly that TRβ is critical for intestinal remodeling, from the reduction in intestinal length to the two major cellular changes, stem cell formation/proliferation and larval epithelial apoptosis via regulation of direct T3 target genes in the intestine. Our results have revealed the complications in studying gene function in vivo and in interpreting developmental outcomes due to gene-editing. It further highlights the advantage of the anuran metamorphosis model for investigating adult organ formation during postembryonic development.

Materials and methods

Experimental animals

Wild type adult X. tropicalis were purchased from Nasco or raised in the laboratory. Embryos and tadpoles were staged according to [69]. TRβ mutant frogs [TRβ+/−] and [TRβ−/−] were reared in the 9 l plastic container until sexual maturation. All animal care and treatments were performed as approved by the Animal Use and Care Committee of Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health.

Generation of TRβ knockout Xenopus tropicalis animals by using CRISPR-Cas9 genome editing technology and genotyping

CRISPR-short guide RNA (sgRNA) was designed as described [60, 70], to target exon 2 of TRβ gene,
A

**Wild type**

|             | Hoechst | EdU |
|-------------|---------|-----|
| **Control** | ![Control_Hoechst](image1) | ![Control_EdU](image2) |
| **2Day T3** | ![2Day_T3_Hoechst](image3) | ![2Day_T3_EdU](image4) |
| **3Day T3** | ![3Day_T3_Hoechst](image5) | ![3Day_T3_EdU](image6) |
| **5Day T3** | ![5Day_T3_Hoechst](image7) | ![5Day_T3_EdU](image8) |

**TRβ(-/-)**

|             | Hoechst | EdU |
|-------------|---------|-----|
| **Control** | ![Control_Hoechst](image9) | ![Control_EdU](image10) |
| **2Day T3** | ![2Day_T3_Hoechst](image11) | ![2Day_T3_EdU](image12) |
| **3Day T3** | ![3Day_T3_Hoechst](image13) | ![3Day_T3_EdU](image14) |
| **5Day T3** | ![5Day_T3_Hoechst](image15) | ![5Day_T3_EdU](image16) |

B

**EdU positive area/Hoechst positive area (%)**

- **Wild type**
- **TRβ(-/-)**

| Condition   | EdU positive area/Hoechst positive area (%) |
|-------------|---------------------------------------------|
| Control     | ![Control](image17)                        |
| 2 day T3    | ![2Day_T3](image18)                       |
| 3 day T3    | ![3Day_T3](image19)                       |
| 5 day T3    | ![5Day_T3](image20)                       |

**Significance**

- ns
- ab
- a
- a'
- b
- c
- *****

**Tukey's post-hoc test**
upstream of the DNA binding domain (Additional file 1: Table S1) (Fig. 1a). CRISPR-sgRNA injected embryos obtained from mating TRα(+/-)TRβ(+/+) and wild type frogs were reared to sexual maturity (F0-generation frogs). A sexually mature F0 frog was mated with a wild type frog, and their offspring were screened to identify tadpoles with wild type TRα by polymerase chain reaction (PCR) method [56] but mutant heterozygous TRβ by sequence analysis [TRα(+/-)TRβ(+/+)] (Fig. 1b). After TRα(+/-)TRβ(+/-) mutants were sexually mature (F1 frogs), female and male mutant frogs were primed with 20 U of human chorionic gonadotropin (Novarel) one day before egg laying. They were then boosted with another injection of 200 U of human chorionic gonadotropin on the second day for natural mating to obtain TRβ knockout [TRβ(−/−)] frogs (F2 generation). The resulting fertilized eggs/embryos were collected and reared for three days at 25 °C to reach the feeding stage (stage 45). The tadpoles were then transferred to a 4-L container and fed.

Tadpoles at indicated ages or stages were anesthetized with MS222 for photography, tail clipping, and body length measurement. For genotyping, tadpole tail tip (about 5 mm or less) was clipped and lysed in 20 µl QuickExtract DNA extraction solution (Epicentre) at 65 °C for 20 min. After incubating at 95 °C for 5 min, 1 µl of the DNA extraction solution was used for genotyping with PCR. A mutant line with a 5 base out-of-frame deletion in TRβ (Fig. 1b) was chosen for further studies. TRβ mutants were identified by PCR analysis of the genomic DNA with the forward primer F, 5′-TCAATGGAACCC TTTGGAAGCTG-3′ and the reverse primer R, 5′-ACAGTTACAGCACATTCCAGGC-3′ for 35 cycles of 94 °C for 10 s, 60 °C for 5 s, 72 °C for 45 s. The PCR products were analyzed by gel electrophoresis and purified by using QIAGEN PCR purification kit (Qiagen), followed by sequencing (Eurofins genomics).

T3 treatment

Randomly selected stage 54 tadpoles were treated with or without 10 nM T3 for 18 h at 25 °C. The tadpole tail tip (about 5 mm or less) was cut for genotyping, and the rest of each animal was frozen in liquid nitrogen. The frozen tadpoles of the same genotype were combined together for RNA extraction. For the long term T3-treatment, wild type and TRβ knockout tadpoles at stage 54 were pooled into separate 4-L plastic containers and treated with 5 nM T3 for 0 (control), 2, 3, and 5 days at 25 °C. Half of the rearing water was replaced with fresh water with or without T3 every day. At least 3 tadpoles in each genotype were picked up and analyzed for each time point of T3 treatment.

RNA extraction and qRT-PCR

The intestine from individual tadpoles was homogenized with RNeasy® Mini Kit 250 (Qiagen). The homogenates from at least three animals of each genotype were combined together for RNA extraction. The RNA concentration was measured by using a NanoDrop (Thermo Scientific). The same amount of RNA from each of the three genotypes (TRβ: wild-type, heterozygous and homozygous mutant) was reverse transcribed with the QuantiTect reverse transcription kit (Qiagen). The cDNA was analyzed by using the SYBR Green qPCR method. The PCR primers for indicated genes and internal control rpl8 were described previously [56, 61] (note that rpl8 mRNA expression were similar in all genotypes; data not shown) (Additional file 2: Table S2). All expression data were normalized against that of the internal control gene rpl8. The expression analyses were performed at least twice, with similar results.

ChIP assay and quantitative PCR

Age-matched stage 54 tadpoles were randomly selected and treated with or without 10 nM T3 for 18 h at 25 °C in 4-L container as described above. They were then anesthetized in ice-water and genotyped by tail clipping. Five tadpoles of the same genotype were pooled together and homogenized for ChIP assay with anti-TR and anti-ID14 (negative control) antibodies as described previously [56, 71, 72]. The immunoprecipitated DNA was analyzed by using TaqMan quantitative PCR (Thermo Fisher Scientific) with gene specific primers/probes for the promoter and exon 5 of TRβ, as previously described [73].
5-Ethynyl-2'-deoxyuridine (EdU) labeling

EdU staining was performed as described [67]. Briefly, 1.25 and 10 μl of 10 mg/ml EdU were injected into stage 54 tadpoles. 30 min after injection, the tadpoles were sacrificed, and the intestine was isolated and fixed in 4% PFA/PBS for paraffin-sectioning. Tissue sections cut at 5 μm were subjected to EdU staining by using the Click-IT Plus EdU Alexa Fluor 594 Imaging kit (Thermo Fisher Scientific). EdU positive areas in epithelium were measured by using ImageJ software (National Institutes of Health).

TUNEL assays

TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling) assays were performed by using In Situ Cell Death Detection Kit (Roche) as described [74]. The fluorescent pictures for different colors and different sections were taken under the same settings and then analyzed by using ImageJ software at the same setting to measure the TUNEL positive cell area.

Methyl green-pyronin Y (MGPY) staining

Tissue sections were stained with MGPY (Muto), a mixture of methyl green, which binds strongly to DNA, and pyronin Y, which binds strongly to RNA, for 5 min at room temperature according to supplier’s instructions. Adult epithelial stem/progenitor cells were intensely stained red because of their RNA-rich cytoplasm [51, 75, 76].

Statistical analysis

Data are presented as mean ± SE. The significance of differences between groups was evaluated by one-way ANOVA followed by Bonferroni multiple comparison test or Student’s t test using Prism 5 (GraphPad Software).

Supplementary information

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Authors’ contributions

YS and YT designed and carried out experiments and interpreted the findings. YS and YBS prepared the manuscript. All authors revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Lazar MA. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev. 1993;14(2):184–93.
2. Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev. 2001;81(3):1097–142.
3. Tata JR. Gene expression during metamorphosis: an ideal model for post-embryonic development. BioEssays. 1993;15(4):239–48.
4. Shi Y-B. Amphibian Metamorphosis: From morphology to molecular biology. New York: Wiley; 1999.
5. Atkinson BG. Metamorphosis: model systems for studying gene expression in postembryonic development. Dev Genet. 1994;15:313–9.
6. Hetzel BS. The story of iodine deficiency: an international challenge in nutrition. Oxford: Oxford University Press; 1989.
7. Freake HC, Oppenheimer JH. Thermogenesis and thyroid function. Annu Rev Nutr. 1995;15:263–91.
8. Franklyn JA, Gammage MD. Thyroid disease: effects on cardiovascular function. TEM. 1996;7:50–4.
9. Silva JE. Thyroid hormone control of thermogenesis and energy balance. Thyroid. 1995;5(6):481–92.
10. Buchholz DR, Paul BD, Fu L, Shi YB. Molecular and developmental analyses of thyroid hormone receptor function in Xenopus laevis, the African clawed frog. Gen Comp Endocrinol. 2006;145(1):1–19.
11. Shi YB, Matsuura K, Fujimoto K, Wen L, Fu L. Thyroid hormone receptor actions on transcription in amphibia: the roles of histone modification and chromatin disruption. Cell Biosci. 2012;2(1):42.
12. Hsia SC, Bedadala GR, Balish MD. Effects of thyroid hormone on HSV-1 gene regulation: implications in the control of viral latency and reactivation. Cell Biosci. 2011;1(1):24.
13. Sato Y, Buchholz DR, Paul BD, Shi Y-B. A role of unliganded thyroid hormone receptor in postembryonic development in Xenopus laevis. Mech Dev. 2007;124:476–88.
14. Paul BD, Fu L, Buchholz DR, Shi Y-B. Coactivator recruitment is essential for liganded thyroid hormone receptor to initiate amphibian metamorphosis. Mol Cell Biol. 2005;25:5712–24.
15. Laudet V, Gronemeyer H. The nuclear receptor FactsBook. San Diego: Academic Press; 2002.
16. Burke LJ, Banerji A. Co-repressors 2000. FASEB J. 2000;14(13):1876–88.
17. Jones PL, Shi Y-B. N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear hormone receptors. In: Workman JL, editor. Current topics in microbiology and immunology: protein complexes that modify chromatin. vol. 274. Berlin: Springer-Verlag; 2003: 237–268.
18. Zhang J, Lazar MA. The mechanism of action of thyroid hormones. Annu Rev Physiol. 2000;62:439–66.
19. Jones PL, Sachs LM, Rouse N, Wade PA, Shi YB. Multiple N-CoR complexes contain distinct histone deacetylases. J Biol Chem. 2001;276(12):8887–91.

20. McKenna NJ, O’Malley BW. Nuclear receptors, coregulators, ligands, and selective receptor modulators: making sense of the patchwork quilt. Ann N Y Acad Sci. 2001;949:3–5.

21. Wong J, Shi YB, Wolfe AP. A role for nuclease assembly in both silencing and activation of the Xenopus TR beta A gene by the thyroid hormone receptor. Genes Dev. 1995;9(21):2696–711.

22. Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988;240:889–95.

23. Tsai MJ, O’Malley BW. Molecular mechanisms of action of steroid/thyroid hormone receptor superfamily members. Ann Rev Biochem. 1994;63:451–86.

24. Gauthier K, Chassande O, Plateroti M, Roux JP, Legrand C, Pain B, Rousset B, Weiss R, Trouillas J, Samarut B. Different functions for the thyroid hormone receptors Thalpa and TRbeta in the control of thyroid hormone production and post-natal development. EMBO J. 1999;18:623–31.

25. Gauthier K, Plateroti M, Harvey CB, Williams GR, Weiss RE, Refetoff S, Willott JF, Sundin V, Roux JP, Malaval L, Hara M, Samarut J, Chassande O. Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. Mol Cell Biol. 2001;21(14):4748–60.

26. Gothe S, Wang Z, Ng L, Kindblom JM, Barros AC, Olsson C, Vennstrom B, Forrest D. Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. Genes Dev. 1999;13(10):1329–41.

27. Wikstrom L, Johansson C, Salto C, Barlow C, Barros AC, Baas F, Forrest D, Thoren P, Vennstrom B. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor. EMBO J. 1998;17:455–61.

28. Fraichard A, Chassande O, Plateroti M, Roux JP, Trouillas J, Dehay C, Legrand C, Gauthier K, Kedinger M, Malaval L, Rousset B, Samarut B. The T3R alpha gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. EMBO J. 1997;16(14):4412–20.

29. Forrest D, Erway LC, Ng L, Altschuler R, Curran T. Thyroid hormone receptor beta is essential for development of auditory function. Nat Genet. 1996;13(3):334–7.

30. Forrest D, Vennstrom B. Functions of thyroid hormone receptors in mice. Thyroid. 2000;10(1):41–52.

31. Ng L, Hurley JB, Dierks B, Riinins M, Salto C, Vennstrom B, Reh TA, Forrest D, Thoren P. Thyroid hormone receptor ensures temporal coordination of tissue-specific metamorphosis. PLoS ONE. 2012;7:e47407.

32. MacDonald WC, Trier JS, Everett NB. Cell proliferation and migration in the stomach, duodenum, and rectum of man: radioautographic studies. Gastroenterology. 1964;46:405–17.

33. van der Flier LC, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241–60.

34. Okada M, Shi YB. Organ-specific requirements for thyroid hormone-inducible expression of Sonic hedgehog correlates with adult epithelial development during remodeling of the Xenopus stomach and intestine. Differentiation. 2001;69:27–37.

35. Shibata Y, Wen L, Miller TC, Su D, Shi YB. Molecular and cytological analyses reveal distinct transformations of intestinal epithelial cells during Xenopus metamorphosis. Cell Biosci. 2015;5:74.

36. Su Y, Shi Y, Stolow M, Shi YB. Thyroid hormone induces apoptosis in primary cell cultures of tadpole intestine: cell type specificity and effects of extracellular matrix. J Cell Biol. 1997;139:1533–43.

37. Shibata Y, Ueda S, Inokuchi T, Amano T, Damjanovski S, Stolow M, Shi YB. Thyroid hormone-induced expression of Sonic hedgehog correlates with adult epithelial development during remodeling of the Xenopus stomach and intestine. Dev Growth Differ. 2007;49(4):317–27.

38. Shibata Y, Erway LC, Ng L, Altschuler R, Curran T. Thyroid hormone receptor alpha ensures temporal coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

39. Shibata Y, Stolow M, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis. Endocrinology. 2015;162:186–98.

40. Shi YB. Thyroid hormone receptor alpha controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

41. Shibata Y, Shiba H, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

42. Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

43. Shibata Y, Shiba H, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

44. Shibata Y, Shiba H, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

45. Shibata Y, Shiba H, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.

46. Shibata Y, Shiba H, Shi YB. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue-specific metamorphosis in Xenopus tropicalis. Endocrinology. 2008;149:5610–8.
65. Nakajima K, Takahashi A, Yaoita Y. Structure, expression, and function of the Xenopus laevis caspase family. J Biol Chem. 2000;275:10484–91.
66. Ishizuya-Oka A, Kajita M, Hasebe T. Thyroid hormone-regulated Wnt5a/Ror2 signalling is essential for dedifferentiation of larval epithelial cells into adult stem cells in the Xenopus laevis intestine. PLoS ONE. 2014;9(9):e107611.
67. Okada M, Shi YB. Cell Proliferation analysis during Xenopus metamorphosis: using 5-ethyl-2-deoxyuridine (EdU) to stain proliferating intestinal cells. Cold Spring Harb Protoc. 2017;2017(9):pdb prot097717.
68. Okada M, Miller TC, Wen L, Shi YB. A balance of Mad and Myc expression dictates larval cell apoptosis and adult stem cell development during Xenopus intestinal metamorphosis. Cell Death Dis. 2017;8(5):e2787.
69. Nieuwkoop PD, Faber J. Normal table of Xenopus laevis. Amsterdam: North Holland Publishing; 1965.
70. Shibata Y, Bao L, Fu L, Shi B, Shi YB. Functional studies of transcriptional cofactors via microinjection-mediated gene editing in Xenopus. Methods Mol Biol. 2019;1874:507–24.
71. Matsuura K, Fujimoto K, Das B, Fu L, Lu CD, Shi YB. Histone H3K79 methyltransferase Dot1L is directly activated by thyroid hormone receptor during Xenopus metamorphosis. Cell Biosci. 2012;2(1):25.
72. Buchholz DR, Ishizuya-Oka A, Shi YB. Spatial and temporal expression pattern of a novel gene in the frog Xenopus laevis: correlations with adult intestinal epithelial differentiation during metamorphosis. Gene Expr Patterns. 2004;4(3):321–8.
73. Buchholz DR, Paul BD, Shi YB. Gene-specific changes in promoter occupancy by thyroid hormone receptor during frog metamorphosis. Implications for developmental gene regulation. J Biol Chem. 2005;280(50):41222–8.
74. Bao L, Roediger J, Park S, Fu L, Shi B, Cheng SY, Shi YB. Thyroid hormone receptor alpha mutations lead to epithelial defects in the adult intestine in a mouse model of resistance to thyroid hormone. Thyroid. 2019;29(3):439–48.
75. Ishizuya-Oka A, Shi YB. Regulation of adult intestinal epithelial stem cell development by thyroid hormone during Xenopus laevis metamorphosis. Dev Dyn. 2007;236:3358–68.
76. Ishizuya-Oka A, Ueda S, Damjanovski S, Li Q, Liang VC, Shi Y-B. Anteroposterior gradient of epithelial transformation during amphibian intestinal remodeling: immunohistochemical detection of intestinal fatty acid-binding protein. Dev Biol. 1997;192(1):149–61.

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