Growth, Development, and Intestinal Remodeling Occurs in the Absence of Thyroid Hormone Receptor α in Tadpoles of *Xenopus tropicalis*

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During development in all vertebrates, thyroid hormone receptors (TRs) are expressed before as well as during and after the peak in plasma thyroid hormone (TH) levels. Previously, we established a role for unliganded TRα in gene repression and developmental timing using tadpoles of TRα knockout (TRαKO) frogs. Here, we examined the role of liganded TRα on growth, development, and intestinal remodeling during natural and TH-induced metamorphosis. Disrupted TRα had little effect on growth during the larval period, but after metamorphosis, TRαKO juveniles grew more slowly than wild-type (WT) juveniles. TRαKO tadpoles developed faster throughout premetamorphosis when TH was low or absent, and despite their decreased responsivity to exogenous TH, TRαKO tadpoles not only were able to complete TH-dependent metamorphosis but also did so earlier than WT tadpoles. In contrast to external morphology, larval epithelial cell apoptosis and adult cell proliferation of intestinal remodeling were delayed in TRαKO tadpoles. Also, TRαKO intestines did not shrink in length to the full extent, and fewer intestinal folds into the lumen were present in TRαKO compared with WT juveniles. Such delayed remodeling occurred despite higher premetamorphic expression levels of TH target genes important for metamorphic progression—namely, *TRβ*, *Klf9*, and *ST3*. Furthermore, the decreased TH-dependent intestinal shrinkage was consistent with reduced TH response gene expression during natural and TH-induced metamorphosis. As in the TRα null mouse model, TRαKO frogs had statistically significant but surprisingly mild growth and development phenotypes with normal survival and fertility. **(*Endocrinology* 158: 1623–1633, 2017)**

In all vertebrates, thyroid hormone (TH) is critical to normal physiology for regulating growth, metabolism, and development (1–3). Nearly all tissues require TH for normal development, and a peak in plasma TH levels occurs in all vertebrates at some point during ontogeny, around birth in mammals, hatching in birds, and metamorphosis in amphibians (4–7). In humans, insufficient levels of TH signaling during this period cause severe mental retardation, with even mild reductions affecting IQ (8, 9). The developmental actions of TH are mediated by TH receptors (TRs), which are nuclear receptor transcriptional factors with dual action in the regulation of TH target genes (1, 10). Unliganded TRs bind to TH response elements of TH target genes and repress gene activation by recruiting corepressors. However, when TH is available, TRs bind to TH, recruit coactivators, and induce target genes. This dependence on TH for TR action led to the dual-function model for the role of TR in the regulation of developmental changes (5, 11, 12). Unliganded TRs early in development act to repress genes important for developmental progression, and upon binding TH, TRs activate those same genes to initiate TH-dependent developmental events.

Understanding the wide-ranging roles of TRs during the experimentally intractable period of perinatal development in humans requires studies in model organisms (13). Use of mouse models to elucidate developmental mechanisms of TH signaling is challenging due to the

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Abbreviations: *Klf9*, Krüppel-like factor 9; NF, Nieuwkoop and Faber; qPCR, quantitative polymerase chain reaction; *rpL8*, ribosomal protein L8; *shh*, sonic hedgehog; *ST3*, stromelysin 3; SVL, snout-to-vent length; TH, thyroid hormone; TR, thyroid hormone receptor; TRαKO, thyroid hormone receptor α knockout; WT, wild-type.

difficulty of observing relatively subtle or cryptic TH-dependent changes and of obtaining samples from fetuses *in utero*. An additional complication is that fetal tissues are constantly exposed to maternal TH through the placenta (4), such that manipulation of fetal TH signaling is difficult to achieve without potentially introducing artifacts from altered maternal TH physiology. Frog metamorphosis has served as an excellent model to study the developmental roles of TRs (2, 13–17). The dramatic TH-dependent molecular and morphological changes that occur during metamorphosis are unrivaled among terrestrial vertebrates, where signaling via TH and their receptors is necessary and sufficient to initiate nearly all developmental events during metamorphosis. Also, being free living, tadpoles afford easy access to observe and/or manipulate them at any developmental time point. Importantly, mechanisms of TH signaling in gene regulation and development are highly conserved between frogs and humans, and TH-dependent stages in tadpoles are comparable to perinatal stages in humans (13). Thus, because frogs are easy to breed and maintain in the laboratory, are the closest vertebrate relatives to humans that have free-living larvae with readily manipulable endocrine signaling, and have all the modern tools of a genetic model system, including a sequenced genome, an ORFeome, transgenesis, and gene knockout technology (18–21), amphibian development is a particularly compelling model system for use in elucidating the actions and mechanisms of TH signaling during development.

Previously, we used TRα-knockout (TRαKO) *Xenopus tropicalis* to show that TRα regulates developmental timing and contributes to tissue responsivity to TH (22, 23). The TALEN-targeted TRα mutation results in truncated proteins lacking a full DNA binding domain with no hinge and ligand-binding ability or known transcriptional activity. TRαKO tadpoles had increased whole-body expression of TH response genes and precocious initiation of metamorphosis due to the lack of TRα-mediated repression in premetamorphic tadpoles and impaired whole-body induction of TH target genes after exogenous TH treatment. It was also shown that lack of TRα led to accelerated growth in TRαKO tadpoles in the early stages of larval development (23). Because frogs are not known to have internal promoters controlling expression of TRΔα transcripts (24, 25), this frog model appears to be most similar to the $TR\alpha^{0/0}$ mouse model lacking all known functional TRα transcripts (26). $TR\alpha^{0/0}$ mice have reduced growth after weaning but not before, and they also have normal survivorship and fertility. In addition, these mice have subtle defects in bone and intestine development with little alteration in plasma TH levels.

In frogs, TRα was expected to play a dominant role in metamorphosis because (1) virtually every tissue has dramatic responses to TH (6), (2) TRα is expressed earlier in stage and at higher levels than TRβ among tissues (27), and (3) transgenic overexpression of dominant negative TRs and dominant negative transcriptional coactivators blocks metamorphic progress (28–31). Our previous study with TRαKO tadpoles during premetamorphosis suggested that TRα may not be as crucial as previously believed, because we saw that the otherwise TH-dependent hindlimb development initiated precociously without TH in TRαKO animals (22). On the other hand, hindlimb development and gill resorption were statistically significantly delayed in TRαKO animals in response to exogenous TH compared with wild-type (WT) tadpoles. Here, we examined the role of TRα in the growth and developmental rate of TRαKO animals during natural and TH-induced metamorphic development. In addition, we assessed the role of TRα in intestine remodeling. Our findings indicate that lack of TRα affected growth after but not before metamorphosis, reduced the larval period duration, and altered the timing of intestinal remodeling events but otherwise appeared to have only mild morphological consequences.

Materials and Methods

Animals and experimental designs

TRα knockout founder animals (22) were bred to obtain embryos by priming the founders with 20 U of human chorionic gonadotropin (Sigma-Aldrich) 14 to 16 hours before boosting with 200 U of human chorionic gonadotropin. Once hatched, the tadpoles were fed (Sara Micron) twice a day, and their water was changed daily. One to two weeks after hatching, tadpoles were sorted into WT or TRαKO groups based on the developmental stage of their hind limbs. Such a "hindlimb phenotype" is a reliable indicator of disruption of both alleles of TRα as confirmed by genomic DNA sequencing (22). For growth and developmental rate studies, 18 tadpoles were randomly selected from the WT and TRαKO tadpoles and divided into six groups, where three tadpoles were reared per 2-L tank. The tadpoles were reared at 27°C on the same shelf, and tank arrangement was haphazardly switched daily to avoid tank effects. In addition, the same amount of food was given to the tadpoles twice daily before and after their water was changed. Very low mortality occurred, where 2 of 18 WT and 1 of 18 TRαKO tadpoles died during the growth and developmental rate studies. Snout-to-vent length (SVL) and Nieuwkoop and Faber (NF) stage (32) were measured every 4 to 5 days. Also, time to and SVL at NF66 were recorded, and SVL at 28 days after metamorphosis was recorded. Sex was not determined, but the sexes do not differ in SVL until after 10 weeks after metamorphosis (33). Intestine length measurements across developmental stages and after metamorphosis were measured from the duodenum (bile duct conjunction) to the end of the ileum, and these measurements were divided by SVL to produce relative intestine length values. Some WT and TRαKO tadpoles were reared in 0, 2, or 10 nM triiodothyronine (Sigma-Aldrich)

without feeding or in 1 mM methimazole (Sigma-Aldrich) with feeding, all with daily water changes and triiodothyronine and methimazole replacement. The use of animals in these studies was approved by the University of Cincinnati Institutional Animal Care and Use Committee (protocol 06-10-03-01).

Reverse transcription quantitative polymerase chain reaction

Intestines were collected at multiple developmental stages or after triiodothyronine treatments, flushed of contents using a needle and syringe filled with 60% phosphate-buffered saline, snap frozen on dry ice, and stored at −80°C until used. Total RNA was extracted using TRI Reagent (Molecular Research Center) from whole bodies or intestines flushed of contents. In total, 1 μg total RNA was used for complementary DNA synthesis (Applied Biosystems), and 1 μL complementary DNA was used for each quantitative polymerase chain reaction (qPCR) (Fisher Scientific) with TaqMan primer/probe sets (Applied Biosystems) on a 7300 Real Time PCR System with default reaction conditions (50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 10 seconds and 60°C for 1 minutes) (Applied Biosystems). Primer/probe sets for *TRβ*, Krüppel-like factor 9 (*Klf9*), stromelysin 3 (*ST3*), and ribosomal protein L8 (*rpL8*) were used as described in the previous study (22). SYBR green qPCR was conducted with default reaction conditions for sonic hedgehog (*shh*) and *rpL8* (Maxima SYBR green; ThermoFisher). The primer sets designed for SYBR green qPCR were used for *shh* (forward: 5′-AAGACG-AGGAGAACACC-GGAGC; reverse: 5′-CAACCCTCCGTGACCCGCAG) and *rpL8* (forward: 5′-CCCTCAACCATCAGGAGAGA; reverse: 5′-TCTT-TGTACCACGCAGACGA). A single dissociation curve was obtained in all SYBR green qPCR reactions. Four to six samples were used for each group, and a no-template control was used for each gene to monitor for polymerase chain reaction product contamination. Samples were normalized to the reference gene *rpL8*, which is not induced by TH and is stable over the stages examined herein (34, 35). The qPCR results were analyzed by using the ddCt method (36).

Histological analysis

Some heterozygous and biallelic mutant TRα tadpoles were reared to adulthood and bred to obtain offspring with the expected 50/50 ratio of tadpoles with the WT phenotype (*i.e.*, heterozygous for a TRα mutation) and with the hindlimb phenotype (*i.e.*, TRαKO), and we harvested intestines from heterozygous animals (NF61, NF66) and homozygous TRαKO animals (NF60, NF61, NF62, NF63, NF66) (n = 5 per stage per genotype). A large portion of the intestine was collected around the bile duct, flushed of contents using a needle and syringe, and fixed in 95% ethanol on ice for 4 hours to overnight with shaking. A 2- to 4-mm tissue fragment just posterior to the bile duct conjunction was isolated and embedded in paraffin blocks for sectioning. Sections cut at 5 μm were stained with methyl green/pyronine Y (Muto) or hematoxylin and eosin. To detect cellular proliferation, some sections were immunostained with an anti–proliferating cell nuclear antigen monoclonal antibody (1:1000; Novocastra) at 4°C for 1 hour. They were then visualized by sequential incubation with streptavidin-biotin-peroxidase complex (Nichirei) and 3,3′-diaminobenzidine (DAB)/H_2O_2. For histological measurements (number of intestinal folds, height of folds, and cross-sectional diameter of intestine), five samples were examined for each group, and three cross sections were randomly selected for each sample. The height of five folds from the highest was measured in each section because newly formed folds are very small in height.

Statistical analysis

The significance of the observed differences between WT and TRαKO groups was determined using Student *t* test with α = 0.05. Error bars represent mean ± standard error.

Results

Growth and development in TRαKO tadpoles

To determine the effect of nonfunctional, truncated TRα on overall growth and development during the larval period, we measured size and stage every 4 to 5 days from the beginning of feeding through completion of metamorphosis. As observed previously (23), we found statistically significant differences in tadpole growth only during the first 2 to 3 weeks of the larval period when tadpoles were NF48 to NF52, before detectable plasma TH is present at NF55 (37) [Fig. 1(a)]. After metamorphosis, WT but not TRαKO juveniles grew statistically significantly during 28 days [Fig. 1(b)]. For development rate, we expected (1) accelerated development in premetamorphic TRαKO tadpoles with low or no TH in the blood due to lack of repression from TRα and (2) delayed development after TH is measureable in the blood due to impaired tissue responsivity to endogenous TH. Indeed, TRαKO tadpoles reached NF54 9 days earlier than WT tadpoles [Fig. 1(c), day 26 compared with day 35]. Also, to progress from NF54 to NF57 (when plasma TH levels are less than a quarter of their peak levels at NF62), TRαKO tadpoles required 6 more days than their WT siblings, again consistent with expectations. However, to our surprise, TRαKO tadpoles were more advanced in stage at each time point, even from NF57 to NF66 (end of tail resorption), during which time TH levels reach their peak (37). By the end of metamorphosis, the TRαKO tadpoles displayed a normal external phenotypes and completed metamorphic development 5 days earlier than WT [Fig. 1(d)].

Intestine length in TRαKO tadpoles

Upon internal examination of TRαKO animals, we noticed the lengths of TRαKO intestines were longer at NF66 (tail resorption) and shorter 28 days after metamorphosis compared with WT [Fig. 2(a)]. To more precisely compare TRαKO and WT intestine lengths, we examined relative intestine length throughout the larval period (NF52 to NF66), including stages of intestinal remodeling (NF60 to NF66) [Fig. 2(b)]. Relative intestine lengths between WT and TRαKO tadpoles were similar until NF58, except for NF54. After NF58, WT intestine

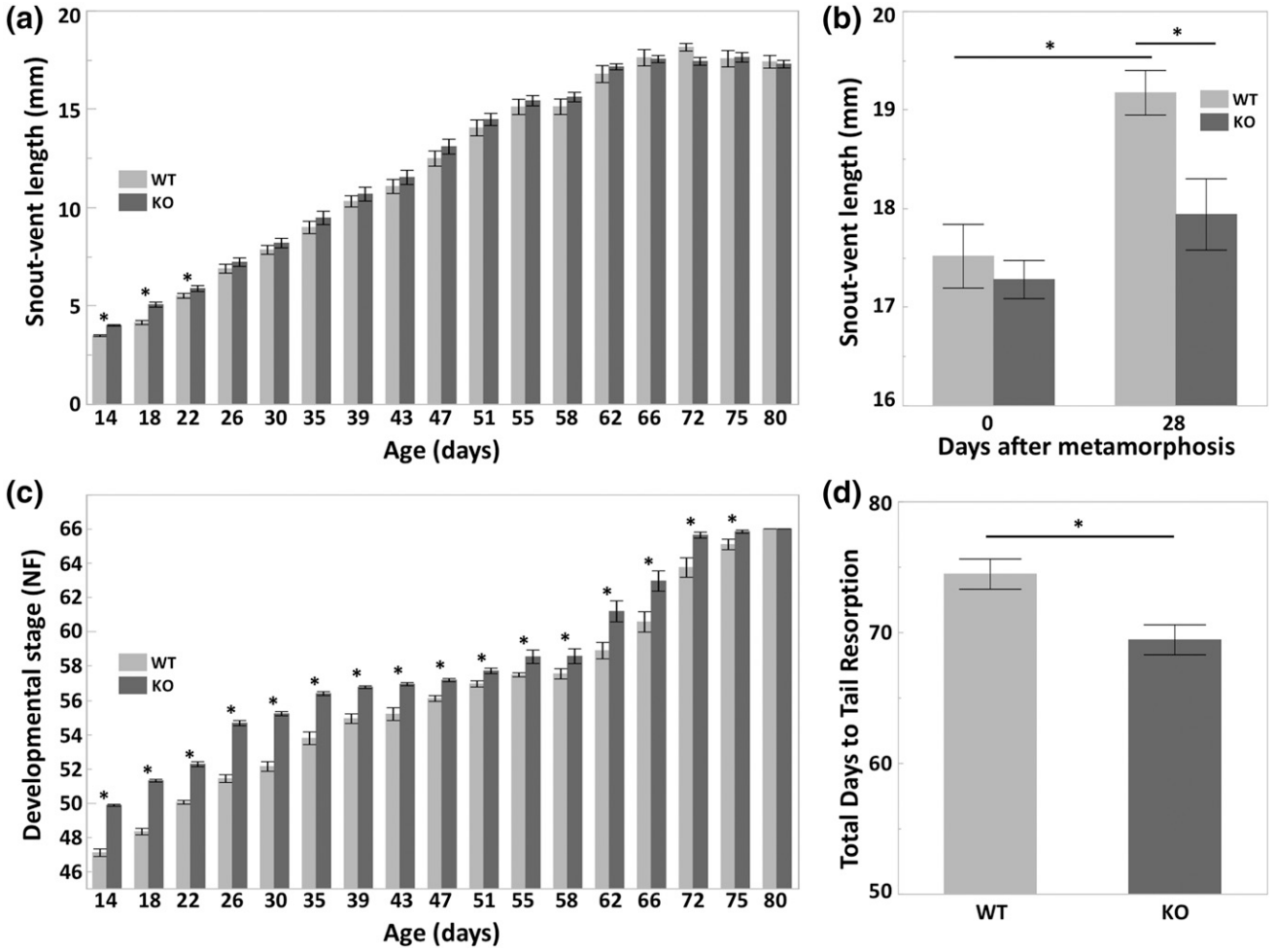

Figure 1. Altered development but not growth in TRαKO tadpoles. (a) Snout-vent length was virtually identical between WT and TRαKO tadpoles throughout the larval period except during the first 3 weeks of age. (b) At NF66 (tail resorption), WT and TRαKO juveniles were the same size, but WT juveniles grew more than TRαKO to become statistically significantly larger by 4 weeks. (c) TRαKO tadpoles developed faster to achieve NF54 (beginning of TH-dependent development), required more time to reach NF57 (completion of hindlimb development), and then developed faster again to achieve NF66 (tail resorption). (d) Over the entire larval period, TRαKO animals reached tail resorption significantly earlier, by 5 days, than WT animals. Tadpoles were reared in six groups of three tadpoles for each WT and TRαKO genotype (n = 18) and measured every 4 to 5 days throughout the larval period. Day of tail resorption (NF66) was recorded for each individual. Statistically significant differences between WT and TRαKO for each day and stage are indicated by asterisks and were determined by the Student *t* test, with α set at 0.05.

initiated shortening earlier (NF59), achieving a maximum shortening at NF65 to 25% of the original length. A one-stage delay in shortening was observed in the TRαKO intestine, with a maximum shortening to 36% at NF65. To examine intestinal shrinkage in TH-induced metamorphosis, we treated sibling, premetamorphic tadpoles at the same age but different stage when intestines are expected to be comparable (WT at NF54 and TRαKO at NF56) with exogenous TH. As in natural metamorphosis, the TRαKO intestine did not decrease in length as much as in WT [Fig. 2(c)].

TH response gene expression in TRαKO intestine

We next determined natural and TH-induced levels of four direct TH response genes—*TRβ*, *Klf9*, *ST3*, and *shh*—in WT and TRαKO intestines to examine potential TH-related impairments at the molecular level (Fig. 3). In premetamorphosis with minimal TH levels, *TRβ*, *Klf9*, and *ST3* expression but not *shh* expression was higher in TRαKO compared with WT tadpoles, as we found previously for whole body (22). Exogenous TH treatment induced *TRβ*, *Klf9*, *ST3*, and *shh* to a significantly lower extent in TRαKO compared with WT intestines, although *shh* was not significantly induced in TRαKO animals. For natural metamorphosis (Fig. 4), a peak in expression of the TH target genes occurred in TRαKO and WT intestines at NF61 and was significantly lower (about one-third that of WT) for *ST3* expression. For *TRβ*, *Klf9*, and *ST3*, there was a delay in returning to premetamorphic levels. Also, for *TRβ* and *Klf9*, there was a delay in increased expression before metamorphic climax at NF61. For *shh*, expression levels were much

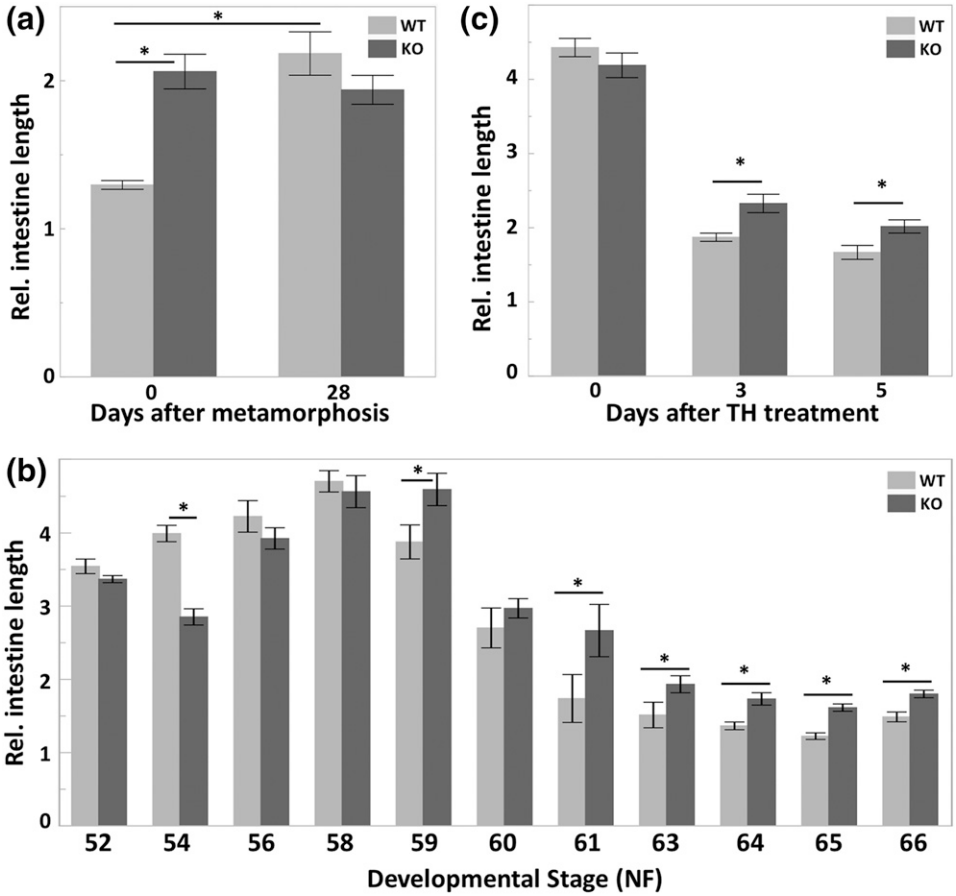

Figure 2. Altered intestine length in TRαKO animals. (a) Relative intestine length of TRαKO animals was longer at NF66 (tail resorption) and shorter in 4-week juveniles compared with WT animals. Intestine length was measured from the duodenum (bile duct conjunction) to the end of the ileum and normalized by snout-vent length. Sample size was six to nine per genotype per time point. (b) Relative (Rel.) intestine length of TRαKO animals was similar to WT prior to intestine remodeling (NF58). Then, WT intestines began shortening earlier than TRαKO intestines by one stage (NF59 vs NF60), and TRαKO intestines failed to shorten to the same extent as WT by the end of intestinal remodeling (~NF65 to NF66). Sample size was four to seven per genotype per stage. (c) After TH treatment, relative intestine length in TRαKO animals also shrank less than WT animals. Tadpoles at the same age when intestines are expected to be comparable but different stages (WT at NF54 and TRαKO NF56) were treated with 10 nM triiodothyronine for 0, 3, and 5 days. Samples size was four to seven per genotype per time point. Statistically significant differences between WT and TRαKO for each day and stage are indicated by asterisks and were determined by the Student *t* test, with α set at 0.05.

higher in WT at NF62 compared with TRαKO but reversed at NF66. To generalize, the expression profiles of TH response genes in TRαKO intestines were lower and shifted later in development compared with WT.

Histological analysis of TRαKO intestine

We determined the cellular effects of altered gene expression in intestinal remodeling by histological analysis using methyl green/pyronine Y staining, which discriminates between larval cells undergoing apoptosis and proliferating adult epithelial progenitors (38). The latter cells are intensely stained red because their cytoplasm rich in RNA is stained with pyronin Y, whereas the former cells become more weakly stained toward their death. The stages of intestinal remodeling have been well characterized in WT intestines stained with methyl green/pyronine Y, where the larval intestinal epithelium is replaced by the adult epithelium via larval cell apoptosis and proliferation of adult cell progenitors derived from a small number of dedifferentiated larval epithelial cells (39, 40). Histological sections in TRαKO and WT intestines during natural metamorphosis revealed distinctly (*i.e.*, intensely) stained nests of proliferating adult precursor cells [Fig. 5(a–c), arrowheads]. Adult precursor cells become flanked by weakly stained apoptotic larval epithelial cells at NF61 in WT animals [Fig. 5(c)]. At NF61, larval cells in TRαKO intestines did not yet have the weakly stained cytoplasm of apoptotic larval cells [Fig. 5(a)], and only by NF63 did the TRαKO intestine histologically match that observed in WT intestines [Fig. 5(b) compared with Fig. 5(c)]. Despite this delay in remodeling, the WT and TRαKO intestinal histology converged by NF66 (end of tail resorption) to achieve similar adult histology and degree of cell proliferation concentrated in the troughs of the intestinal folds

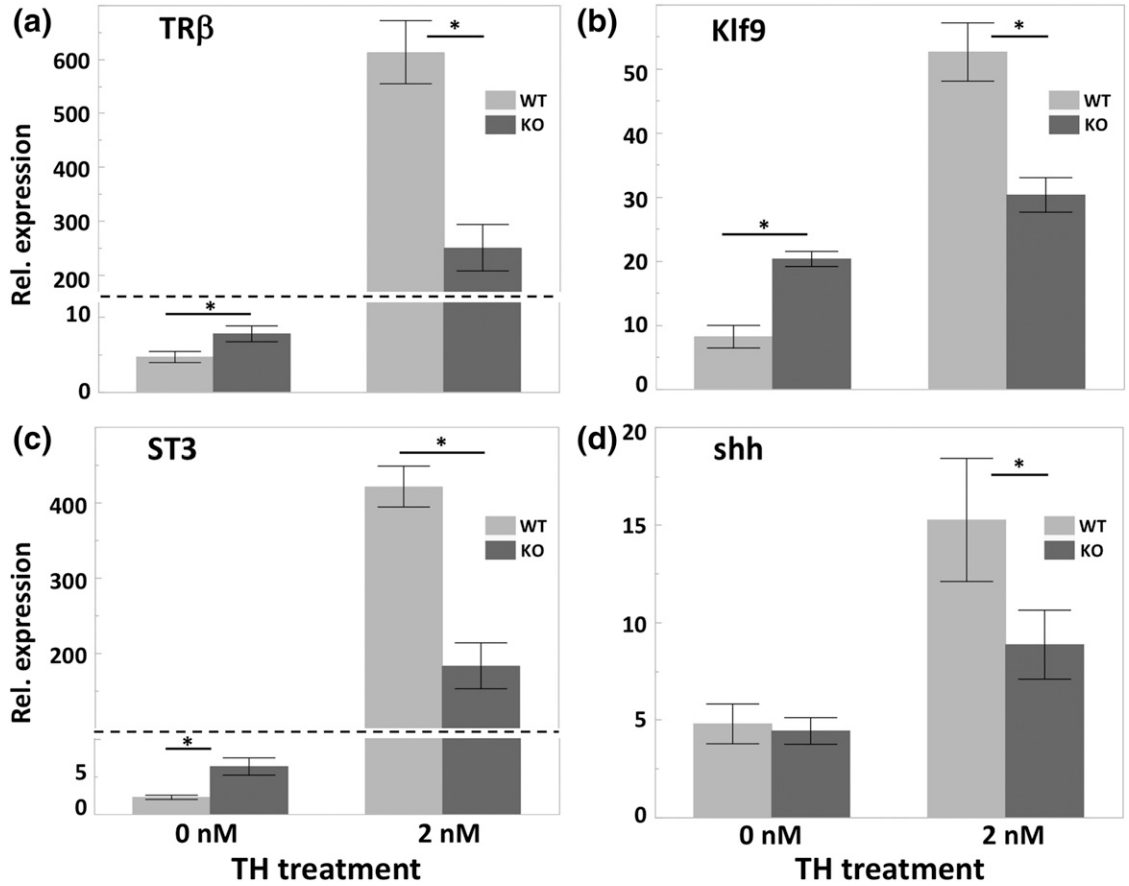

Figure 3. Altered induction of TH target genes in TRαKO animals. Expression levels of (a) *TRβ*, (b) *Klf9*, (c) *ST3*, and (d) *shh* messenger RNA were measured by qPCR from intestines from premetamorphic (NF50 to NF52) WT and TRαKO tadpoles treated with or without 2 nM triiodothyronine for 24 hours. Expression levels of *TRβ*, *Klf9*, and *ST3* were higher in TRαKO animals in the absence of triiodothyronine, but induced levels of those genes and *shh* were higher in WT animals. Values were normalized using the reference gene *rpL8*. Samples size was four to six per genotype per treatment for each gene. Statistically significant differences between WT and TRαKO for each treatment of each gene are indicated by asterisks and were determined by the Student *t* test, with α set at 0.05. Rel., relative.

[Fig. 5(d–g)]. We looked for more subtle differences between WT and TRαKO intestines by quantifying the intestinal histological structure in cross sections potentially indicative of impaired production of adult epithelial cells—namely, intestine diameter and height and number of folds into the lumen [Fig. 5(h)]. We found that the number of intestinal folds was significantly reduced in the TRαKO intestines at NF66 with otherwise similarly sized folds and cross-sectional diameter.

Effects of chemical thyroidectomy on TRαKO tadpoles

From our previous study (22), we found that hind limbs in TRαKO tadpoles developed precociously even in the absence of TH by rearing tadpoles in methimazole beginning at feeding (NF45) prior to thyroid follicle formation. Here, we reared WT and TRαKO tadpoles in methimazole and examined them after 3 to 6 months of treatment, time points at which all their untreated siblings had completed tail resorption. As previously known, WT tadpoles treated continuously with methimazole failed to progress beyond the premetamorphic stage NF54 [Fig. 6(a)]. In methimazole-treated TRαKO tadpoles, forelimb emergence occurred, and hindlimb development continued to completion, although limb elongation was reduced [Fig. 6(b)]. In addition, skin development proceeded beyond the larval condition by forming iridiphores (shiny gold cast to skin coloration) where adult skin formation occurs [Fig. 6(b), black arrows]. In addition, intestinal development proceeded beyond the larval condition in TRαKO tadpoles by forming large infoldings into the intestinal lumen, although the typhlosole was still evident and the muscle layer remained thin as in tadpoles [Fig. 6(c)].

Discussion

Using TRαKO founder animals from our previous study (22), we examined the role of TRαKO during metamorphosis in *X. tropicalis*. We hypothesized that TRαKO tadpoles would fail to metamorphose completely or at least require more time to accomplish developmental

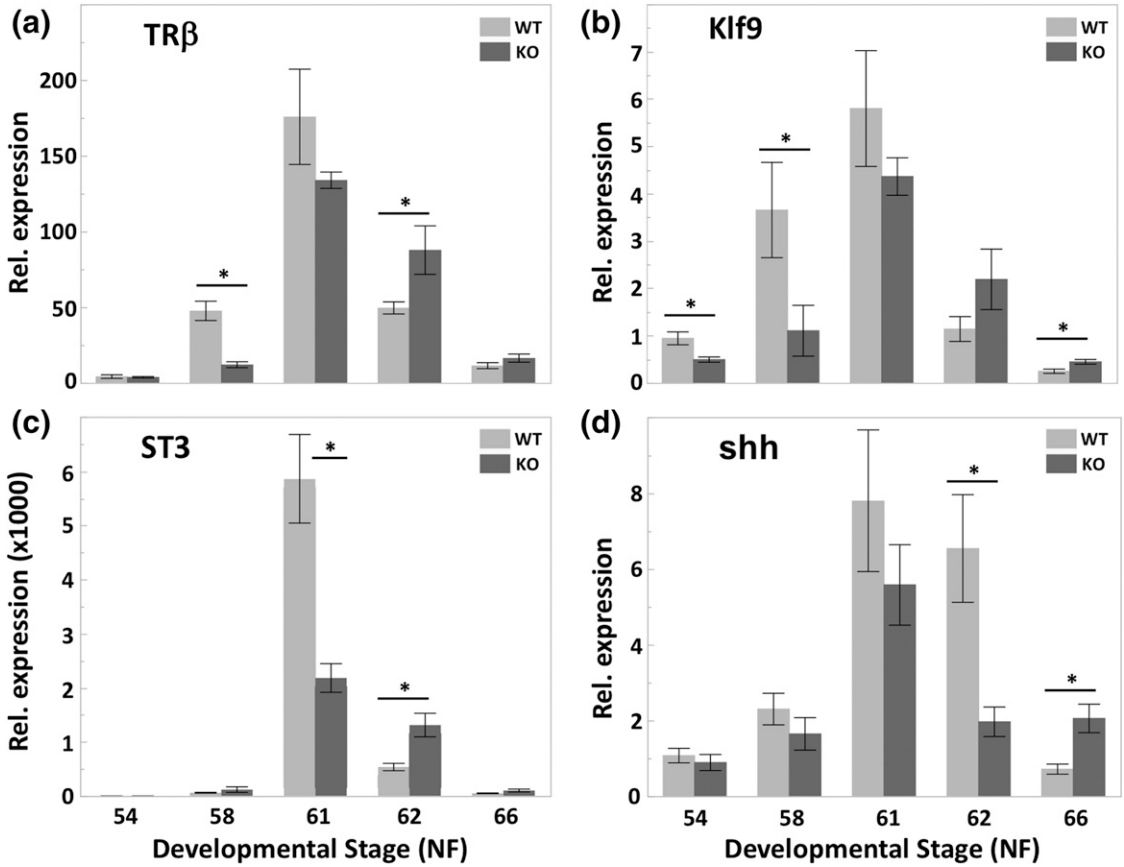

Figure 4. Altered developmental expression profile of TH target genes in TRαKO animals. Expression levels of (a) *TRβ*, (b) *Klf9*, (c) *ST3*, and (d) *shh* messenger RNA were measured by qPCR from intestines from tadpoles at the indicated NF stages. Relative (Rel.) to WT, increases in expression levels were delayed in *TRβ* and *Klf9*, and peak expression was lower for *ST3* and narrower for *shh*. In addition, delayed reduction of expression occurred for all genes after peak levels had been reached. Values were normalized using the reference gene *rpL8*. Sample size was four to five per genotype per NF stage for each gene. Statistically significant differences between WT and TRαKO for each NF stage for each gene are indicated by asterisks and were determined by the Student *t* test, with α set at 0.05.

events based on our previous results showing that responsivity to exogenous TH was dramatically reduced in premetamorphic TRαKO tadpoles. However, we observed that during developmental stages NF48 to NF57, when stages are based on hindlimb development, TRαKO tadpoles actually advanced in stage more rapidly until development of WT tadpoles nearly caught up at NF57 ($\sim$ day 51), when hindlimb development, but not elongation, is complete. After NF57, TRαKO tadpoles again advanced in stage more rapidly than WT tadpoles to an earlier completion of metamorphosis at tail resorption, NF66. This surprising result, that TRαKO tadpoles were not only able to complete metamorphosis but also developed faster than WT tadpoles, was not predicted based on reduced responsivity of TRαKO tadpoles to exogenous TH (22, 23). Growth, on the other hand, was hardly affected until after metamorphosis, when TRαKO juveniles grew more slowly than WT.

These growth and development results in TRαKO frogs are reminiscent to those found in mice lacking all functional TRα transcripts (TR$\alpha^{0/0}$) (26). In TR$\alpha^{0/0}$ mice, growth was reduced after, but not before, weaning, a time during which many TH-dependent developmental events occur (41). Embryonic and fetal development occur normally in TR$\alpha^{0/0}$ mice, although overall developmental timing and timing of birth comparisons were not recorded. In addition, equivalent survivorship and fertility were observed in TR$\alpha^{0/0}$ and WT mice (26). Similarly in frogs, larval period mortality was the same for WT and TRαKO tadpoles, and we reared greater than 15 TRαKO frogs of both sexes to adulthood and obtained viable F2 offspring when crossed with each other or with WT adults. The lack of known internal promoters or TR$\Delta\alpha$ isoforms in the TRα locus in frogs as well as evolutionary conservation of TH control of development between frogs and mammals may explain the high degree of similarity between TRαKO frogs and TR$\alpha^{0/0}$ mice (13, 25).

During tadpole metamorphosis, relative intestinal length dramatically decreases in response to TH to accomplish the transition from an herbivorous larval to a carnivorous adult diet (6, 39). We found that the delayed intestinal remodeling and incomplete shrinkage in TRαKO

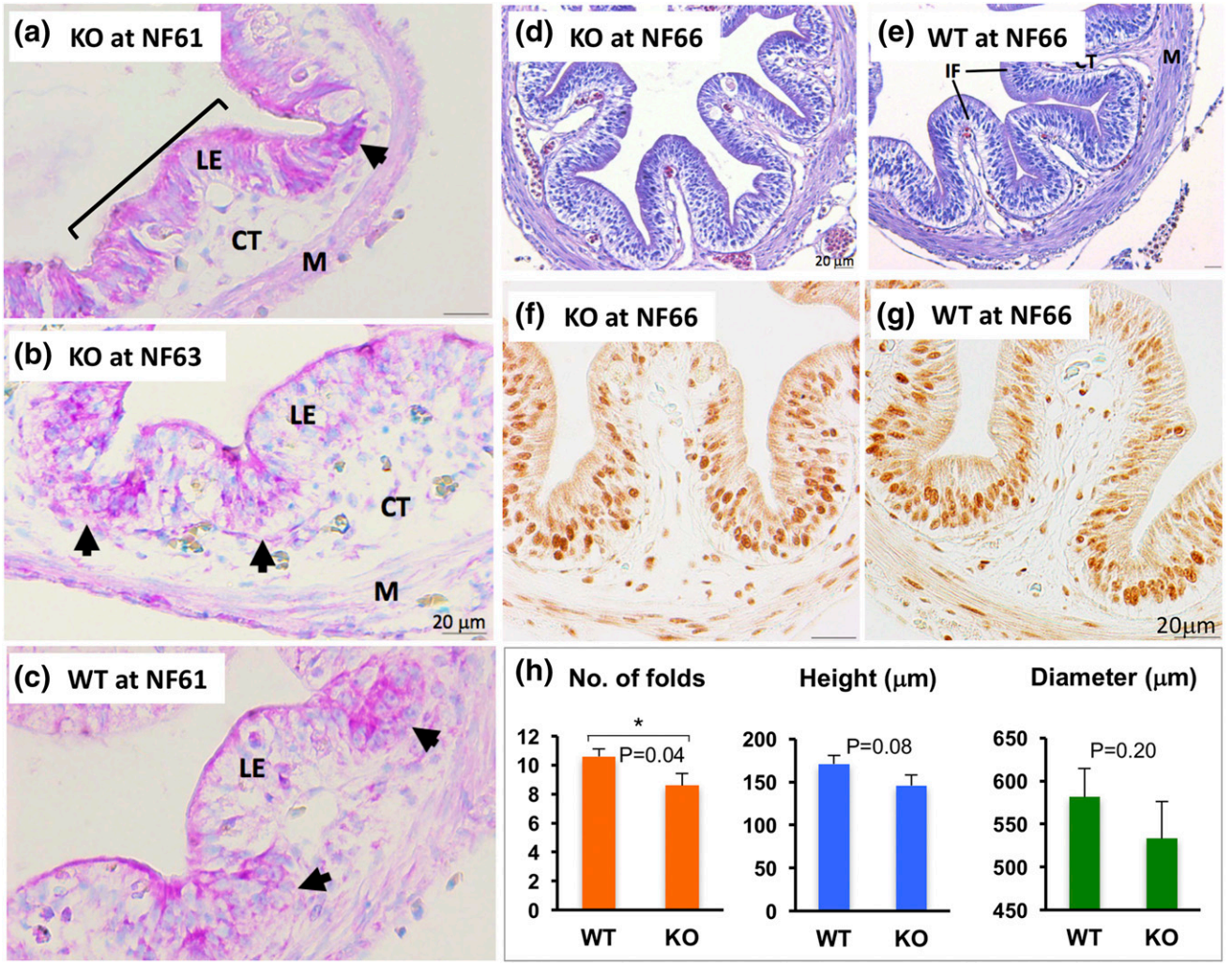

Figure 5. Delayed intestinal remodeling in TRαKO animals. (a–c) WT and TRαKO intestines at the indicated stages (NF) were stained with methyl green/pyronine Y. Dark areas (arrowheads) indicates adult proliferating cells between larval epithelium (LE and bracket in a). CT, connective tissue; M, intestinal smooth muscle. (d, e) WT and TRαKO intestines at the end of tail resorption (NF66) were stained with hematoxylin and eosin and appear very similar. IF, intestinal folds. (f, g) WT and TRαKO intestines at the end of tail resorption (NF66) were processed for immunohistochemistry using an antibody against the proliferating cell marker, proliferating cell nuclear antigen, and again WT and TRαKO appear very similar. Histological images show paraffin sections at 5 μm with scale bars all showing 20 μm, and images in each panel are representative of five individuals examined per genotype per NF stage. (h) The number of intestinal folds into the lumen was smaller in TRαKO compared with WT, but the height of the folds and the intestinal diameter were not significantly different. Statistically significant differences for each histological measurement are indicated by asterisks and were determined by the Student *t* test, with α set at 0.05.

animals were mirrored at the level of gene expression, where the four TH response genes tested—namely, *TRβ*, *Klf9*, *ST3*, and *shh*—had reduced expression in natural and TH-induced metamorphosis. The well-characterized roles of these genes in intestine metamorphosis help explain the impaired intestinal remodeling in TRαKO animals. *TRβ* is at the top of the TH gene regulation cascade (2), where higher TR expression levels increase tissue sensitivity and responsivity to TH (42, 43). Conversely, the lower *TRβ* levels achieved in TRαKO intestines likely reduced responsiveness to TH, contributing to delayed remodeling and reduced intestinal shrinkage. *Klf9* acts as an accessory factor to increase TH-dependent induction of *TRβ* (44), such that reduced *Klf9* expression observed in TRαKO animals would decrease *TRβ* levels and thus decrease intestine responsivity to TH. *ST3* and *shh* contribute to larval intestinal epithelial cell apoptosis and adult epithelial cell proliferation, respectively (45, 46). The dramatically reduced expression of *ST3* in TRαKO animals likely contributed to the delay in larval cell apoptosis because transgenic overexpression of *ST3* promotes larval cell apoptosis, whereas *ST3* inhibitors have the opposite effect (47). Sonic hedgehog may be the least affected by the TRα gene disruption as its premetamorphic levels were not derepressed, and its levels during natural metamorphosis were not significantly different from WT until NF62. Nevertheless, the altered *shh* levels in TRαKO animals may have contributed to the significantly reduced intestinal folds seen in TRαKO animals perhaps via decreased production of adult epithelial progenitors (46), although

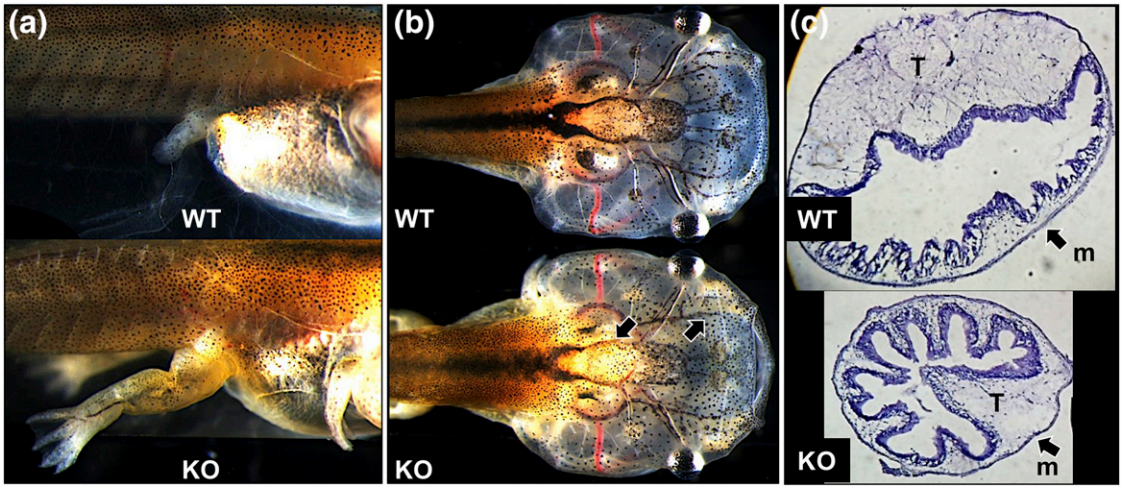

Figure 6. Incomplete inhibition of metamorphosis by methimazole in TRαKO animals. Tadpoles were immersed continuously in 1 mM methimazole beginning at feeding (NF45) to time points after which all untreated siblings had undergone natural metamorphosis. (a) After 13 weeks in methimazole, hindlimb development was blocked at NF54 in WT animals, but apparently complete hindlimb outgrowth, forelimb emergence, and complete toe differentiation (NF57) occurred in TRαKO animals. (b) After 13 weeks in methimazole, developmental progression toward opaque adult skin was observed of TRαKO tadpoles, where ultimobranchial bodies (left arrow) and jaw cartilage margins (right arrow) are partly obscured (covered by shiny gold-colored iridiphores seen in color). Images are representative of eight WT and four TRαKO individuals. Scale bar: 1 mm. (c) After 6 months in methimazole, intestinal cross sections were larval in character as seen by thin muscle layer (m) and typhlosole (T), although TRαKO intestines developed infoldings that resemble those in juvenile intestines (n = 2).

we were not able to measure such a difference in cell proliferation between TRαKO and WT animals.

Despite the altered TH response gene expression, delayed remodeling, and reduced shrinkage, the intestines achieved nearly normal adult intestinal structure by the end of metamorphosis. The slight effects observed in the overall final length of the remodeled intestine with fewer intestinal folds are reminiscent of the similar mild intestinal phenotype in TR$\alpha^{0/0}$ mice after TH-dependent intestinal maturation at weaning (26). The length difference between TRαKO and WT intestines was gone by 28 days after metamorphosis. Interpretation of the juvenile intestine length differences in WT and TRαKO animals is difficult because of potentially confounding explanations. Poor intestine organ growth and/or poor neural control of feeding and appetite affecting overall body growth, both of which are potentially altered in TRαKO animals, may explain juvenile intestine length differences. Our focus on intestinal development up to tail resorption at NF66 when growth in WT and TRαKO animals was similar does not have this complication.

Our studies on TRαKO animals enrich the dual-function model by providing further insights into the role of TRs in development. In the hind limb, precocious development in TRαKO animals is consistent with the role of unliganded TRα acting as a repressor early in development when plasma TH is minimal (5, 11, 12). Surprisingly, prolonged (more than 8 weeks) methimazole treatment showed that the low, de-repressed levels of TH response genes important for metamorphosis acting for a relatively long period of time were sufficient to accomplish limb development even in the absence of TH in TRαKO tadpoles. Previously, it was believed that TH response gene induction was required for all metamorphic events, including limb development.

Thus, hindlimb development may stand as an exception to the dual-function model, at least in animals with disrupted TRα. In the intestine, de-repression of TH response genes prior to circulating TH and impaired induction of TH response genes in the presence of TH are consistent with the dual-function model at the level of gene expression (5, 11, 12). However, delayed rather than precocious initiation of intestinal remodeling was observed. Long periods of de-repressed levels of TH response genes in the intestine (6 months in methimazole) were not sufficient to bring about adult intestinal histology, suggesting that other mechanisms in addition to TRα- mediated repression are acting in premetamorphic intestine to keep metamorphic genes turned off or that TH response gene induction is required for intestinal remodeling. Our results give further insight into the mechanisms of tissue damage in hypothyroid conditions during development, at least for the hind limb. When there is no TH, it is the presence of TR that causes impaired development, because in methimazole-treated tadpoles, the limbs develop to completion in TRαKO rather than being halted at the paddle stage as in WT animals. A comparable situation was observed in mice, where deletion of TRα1 prevented the cerebellar retardation in hypothyroid conditions (48). Our results from the intestine are equivocal because of the uncharacterized involvement of TRβ.

Different effects on hindlimb development and intestinal remodeling in TRαKO tadpoles indicate that the

effect of disrupted TRα is not the same for all tissues, and perhaps such differences among tissues may not be unexpected due to tissue-specific expression levels of TRβ available to compensate for the loss of TRα. Hindlimb development is dominated by TRα, where TRα is highly ubiquitously expressed and TH-dependent TRβ expression is localized to early chondrocytes (27, 49). Thus, lack of TRα-mediated repression and consequent precocious development was apparently not counteracted by wild-type TRβ-mediated repression. The intestine is likely to be substantially influenced by both TRα and β because both TRs are highly expressed in the intestine compared with mostly TRα in the hind limb (50). The delay in TRαKO intestinal remodeling during natural development and predominantly larval intestinal histology after methimazole treatment implies a substantial or at least compensatory role for TRβ.

In other organs, knowledge about the expression of TRα vs TRβ is not always available, but adult skin iridiphore development in methimazole-treated TRαKO tadpoles predicts that skin also has high levels of TRα compared with TRβ, as do hind limbs. Gills and tail may reveal yet another case. TRβ plays a dominant role in those organs (51), and thus, not surprisingly, gills and tail did not show precocious development during natural development or advancements in stage after methimazole treatment. Interestingly, accelerated external development occurred to achieve earlier metamorphic completion in TRαKO tadpoles. Perhaps gills and tail, like hind limb, had some degree of increased TH response gene expression due to lack of repression by TRα, thereby giving them a head start in developmental progression that can be capitalized on by wild-type TRβ. Alternatively, it is possible (because it has not been measured thus far) that TH levels may be higher during metamorphosis in TRαKO tadpoles, enabling more rapid development. However, it is important to note that the $TR\alpha^{0/0}$ mice had no change in plasma TH levels (26). In any case, our results confirm that TRα signaling contributes to developmental coordination among tissues, where disrupted TRα led to precocious limb development but delayed intestine development.

It is possible that knockout frogs lacking both TRα and TRβ may allow metamorphic progress in other organs, as occurs in the hind limb, due to a complete lack of TR-mediated TH response gene repression. Alternatively, TH response gene induction may indeed be required for metamorphic progression in many or most organs, such that de-repressed levels of TH response genes, even if present for an extended period of time, would not be sufficient to accomplish morphological change. In mice completely lacking both TRα and TRβ, developmental timing, mortality, and longevity are not altered, but several notable phenotypes are present, including intestine histology, bone maturation, reduced fertility, and liver TH responsiveness (52). Results from the mouse model indicate that TH response gene induction is important for normal mouse development, and the same may be true for most organs in tadpoles as well. Answers await production and analysis of TRα and TRβ double knockout frogs.

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