Thyroid hormone (T3) receptors (TRs) mediate the effects of T3 on organ metabolism and animal development. There are two TR genes, TRα and TRβ, in all vertebrates. During animal development, TRα expression is activated earlier than zygotic T3 synthesis and secretion into the plasma, implicating a developmental role of TRα both in the presence and absence of T3. Using T3-dependent amphibian metamorphosis as a model, we previously proposed a dual-function model for TRs, in particular TRα, during development. That is, unliganded TR represses the expression of T3-inducible genes during premetamorphosis to ensure proper animal growth and prevent premature metamorphosis, whereas during metamorphosis, liganded TR activates target gene transcription to promote the transformation of the tadpole into a frog. To determine if TRα has such a dual function, we generated homozygous TRα-knockout animal lines. We show that, indeed, TRα knockout affects both premetamorphic animal development and metamorphosis. Surprisingly, we observed that TRα is not essential for amphibian metamorphosis, given that homozygous knockout animals complete metamorphosis within a similar time period after fertilization as their wild-type siblings. On the other hand, the timing of metamorphosis for different organs is altered by the knockout; limb metamorphosis occurs earlier, whereas intestinal metamorphosis is completed later than in wild-type siblings. Thus, our studies have demonstrated a critical role of endogenous TRα, not only in regulating both the timing and rate of metamorphosis, but also in coordinating temporal metamorphosis of different organs. (Endocrinology 158:1985–1998, 2017)
knockdown tadpoles of in vivo made use of the newly developed, TALEN-mediated development, we and the Buchholz laboratory previously shown that TRs indeed bind constitutively to the pro-

chromatin-immunoprecipitation (ChIP) assays have available during metamorphosis (35, 42). Consistently, activation of these T3 target genes when T3 becomes mammalian and amphibian species (5, 30, 34–39). Interestingly, Xenopus TRα expression reaches high levels by the end of embryonic development, when a free-feeding tadpole is formed, well before the onset of metamorphosis at stage 54, whereas the TRβ gene has little expression during embryogenesis or pre-

metamorphosis but is strongly activated by T3 during metamorphosis (5, 16, 34, 39–41). These findings suggest that TRα most likely functions as unliganded TR in premetamorphic tadpoles and binds to T3-inducible genes to repress their expression prior to meta-

morphosis, whereas both TRα and TRβ participate in the activation of these T3 target genes when T3 becomes available during metamorphosis (35, 42). Consistently, chromatin-immunoprecipitation (ChIP) assays have shown that TRs indeed bind constitutively to the pro-
moter regions of T3-induced genes in premetamorphic as well as metamorphosing tadpoles (17, 18).

To investigate the role of endogenous TRα during frog development, we and the Buchholz laboratory previously made use of the newly developed, TALEN-mediated in vivo gene mutation technology to generate TRα-knockdown tadpoles of X. tropicalis (43–46), a diploid species that is highly related to the more widely studied X. laevis (34, 47–50). The studies with these F0-generation knockdown animals revealed that the knockdown of TRα enhanced the development of pre-

metamorphic tadpoles while making the tadpoles resistant to T3 treatment, supporting the previously hypothesized roles of TRα. However, the lack of a well-defined, homozygous, total TRα-knockout line prevented the analysis of the role of TRα during natural metamorphosis in these earlier studies. Here, using F0 knockdown animals, we have generated TRα–total knockout animals to investigate the role of TRα in the development of X. tropicalis. Our results have confirmed the earlier observations with the knockdown animals; that is, the homozygous animals initiate metamorphosis earlier but metamorphose more slowly than their wild-type and heterozygous siblings. More importantly, we have several findings to report. First, the total removal of TRα has no observable effect on embryogenesis. Second, and surprisingly, the animals lacking TRα are able to complete metamorphosis, as judged by external morphology (complete resorption of the tail) within an apparently similar developmental time period as the wild-type and TRα heterozygous animals. Last, the knockdown also alters the temporal coordination of organ-specific transformations: limb formation is precocious, but intestinal metamorphosis is delayed in the knockdown animals compared with the wild-type and heterozygous animals. Thus, TRα, although nonessential, plays a critical role in regulating metamorphosis timing and rate as well as tissue-specific temporal transformations of different organs.

Materials and Methods

Animal rearing and staging

Wild-type adult X. tropicalis were purchased from Nasco. Embryos and tadpoles were staged according to the description for X. laevis (51). All animal care and treatments were performed in accordance with guidelines of 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 TRα-knockout tadpoles and genotyping

TALEN-injected X. tropicalis embryos, as described previously (43), 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 TRα heterozygous mutant tadpoles. After TRα heterozygous 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 200 U of human chorionic gonadotropin on the second day for natural mating. 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 2-L container and fed.

Tadpoles were anesthetized with MS222 for photography, tail clipping, and body length measurement. For genotyping, the tadpole’s tail tip or one forelimb digit (for stage-66 animals) was clipped and lysed in 30 to 50 μL QuickExtract DNA extraction solution (Epicentre) at 65°C for 10 to 15 minutes, according to manual instructions. After incubating at 95°C for 5 minutes, 2 μL of the DNA extraction solution was immediately used for genotyping polymerase chain reaction (PCR). For the F1 animals, genotyping was performed by PCR amplification of the TALEN-targeted region, following by sequencing. This led to the identification of heterozygous F1 animals with out-of-frame mutations. The studies presented here were based on one F1 mutant line. Genotyping the offspring of this F1 line was subsequently performed by PCR. The forward primer 5'-ACATCCCCAGCTATCCCCAGCTATG-3' was designed to detect mutated allele, whereas the forward primer 5'-AGCTATCTGGACAAAGACGAGCCG-3' was used to detect wild-type allele. Each of the forward primers was mixed with the same reverse primer 5'-GCAAACATTTTTGCGTCAGAGGCCAC-3' for PCR. For each DNA sample, two sets of PCRs, for detecting
the wild-type and mutant allele, respectively, were carried out to determine the genotype with the same PCR program: 94°C for 30 seconds, 68°C for 60 seconds, then repeated for 35 cycles. The PCR products were analyzed by gel electrophoresis. The specificity of the primers was verified by using mutant and wild-type genome DNA for PCR.

**T3 treatment**

For gene expression analysis, 6-day-old tadpoles were randomly selected and treated with 10 nM T3 for 18 hours at 25°C. The tadpole tail was cut for genotyping, and the rest of each animal was homogenized in 200 μL TRIzol (Life Technologies). The homogenized solutions from tadpoles of the same genotype were mixed together for RNA extraction. For the ChIP assay, 7-day-old tadpoles were genotyped by tail clipping and then reared in 24-well plates; 5 tadpoles for each genotype were pooled together and treated with 10 nM T3 for 18 hours at 25°C.

**RNA extraction and quantitative real-time PCR**

The intestine, tail, or limb was isolated from animals at indicated stages and homogenized in 500 μL of TRIzol per intestine, tail, or limb, respectively. The homogenates of individual tissues from at least three animals, or three whole animals, of each genotype were mixed together for RNA extraction. Total RNA was extracted with Direct-zol RNA MiniPrep (Zymogen), according to the manufacturer’s instructions. The RNA concentration was measured by using a NanoDrop instrument (Thermo Scientific). The same amount of RNA from the three genotypes (wild-type, heterozygous, and homozygous) was reverse transcribed, respectively, with the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out by using the SYBR Green method. The PCR primers for genes TH/bzip, TRβA, Klf9, Gh1, Gh2, and internal control EF1α were described previously (43) (note that EF1α expression is similar in all three TRα genotypes; data not shown). Other primers included forward primer 5'-AAATGCATTGCCGTTGGCAT and reverse primer 5'-GGTTATGTGTGGCGCCTTCG-3' for Tbx4; and forward primer 5'-TGCCTGCA-TAGCAGGTTC-0 and reverse primer 5'-GAGCACATTGG-0 for Tbx5. All expression data were normalized against that of the internal control gene EF1α. The expression analyses were performed at least twice, with similar results.

**ChIP assay and quantitative PCR**

Tadpoles at 7 days of age were randomly selected and anesthetized with MS222. For genotyping, the anesthetized tadpoles were subjected to tail clipping and reared in 12-well plates individually. After genotyping, five tadpoles of the same genotype were pooled together and homogenized for ChIP assay with anti-TR, anti-NCoR, and anti-ID14 antibodies to serve as negative controls, as described previously (50, 52) (Table 1). The immunoprecipitated DNA was analyzed by using TaqMan quantitative PCR (Thermo Fisher Scientific) with gene-specific primers/probes for TRβ promoter, TH/bZIP promoter, and exon 5 of TRβ, as previously described (18).

**Results**

*X. tropicalis* TRα regulates T3 target genes both in the presence and absence of T3 but is not required for embryogenesis

To generate animals lacking functional TRα, we previously showed that microinjecting TALEN messenger RNAs (mRNAs) targeting exon 4 (the third coding exon), which encodes a portion of the DNA binding domain of TRα in one-cell-stage embryos, led to tadpoles (the F0-generation animals) containing mosaic TRα mutations (43). We raised some of these animals to sexual maturity and then crossed them with wild-type animals to generate F1 animals [Fig. 1(a)]. Genotyping of the F1 animals at tadpole stages identified a number of heterozygous F1 animals with out-of-frame TRα mutations. For functional studies, we chose one mutant line, M5, with seven base substitutions and seven-base-pair deletions in the TRα gene encoding a nonfunctional truncated protein lacking the DNA and ligand binding domains. The heterozygous animals from this M5 line were crossed to generate F2-generation TRα total knockout animals, as confirmed by PCR genotyping [Fig. 1(c)].

It has been shown that low levels of TRs are expressed in early embryos, and TRα is strongly

| Peptide/Protein Target | Antigen Sequence (if Known) | Name of Antibody | Manufacturer, Catalog No., and/or Name of Individual Providing the Antibody | Species Raised in; Monoclonal or Polyclonal | Dilution Used | RRID |
|------------------------|----------------------------|------------------|------------------------------------------------------------------|---------------------------------|-----------|------|
| ID14                   | DKVTPKDDGATS-KLH, ETKCRNMDGDEV-MAP | Anti-ID14       | (24, 50)α                                                    | Rabbit; polyclonal               | 1/100     | AB_2636986 |
| TR                     | Recombinant × 1 TRb protein | Anti-TR         | (24, 34, 50)α                                                | Rabbit; polyclonal               | 1/100     | AB_2636985 |
| NCoR                   | Recombinant × 1 NCoR fragment | Anti-NCoR     | (53)α                                                        | Rabbit; polyclonal               | 1/100     | AB_2636987 |

Abbreviation: RRID, Research Resource Identifier.

* Manufactured in the Shi laboratory. Numbers indicate references.
activated during late embryogenesis to reach high levels at the end of embryogenesis, although TRβ expression remains very low until metamorphosis (5, 16, 34, 39–41, 54–57). Furthermore, altering TR function has been shown to affect embryonic development (16, 54, 57). Interestingly, total knockout of TRα has no apparent effect on embryogenesis, given that wild-type, heterozygous, and homozygous TRα-mutant animals all completed embryogenesis to become feeding stage (stage 45) tadpoles at the same time (3 days after fertilization) without any detectable morphological difference (data not shown), suggesting that TRα is not essential for embryogenesis and/or that its role is offset by the low-level expression of TRβ.

TRα mRNA reaches high levels by the end of embryogenesis when the feeding begins (stage 45) (5, 34, 39), and TR is known to bind to the TREs of endogenous target genes and recruit corepressors in premetamorphic tadpoles (12). Thus, to investigate the effect of TRα knockout on premetamorphic tadpoles, we first analyzed TR binding and corepressor recruitment to two well-known target genes, TRβ (58) and the transcription factor TH/bzip (59), in 7-day-old premetamorphic tadpoles by ChIP assay. As shown in Fig. 2(a), both TR binding and the recruitment of the corepressor NCoR to the TREs were drastically reduced in the homozygous TRα-knockout animals compared with the wild-type and heterozygous siblings. The remaining TR binding was likely due to the expression of TRβ in the knockout tadpoles.

Consistent with the reduced TR binding and corepressor recruitment, we also observed that the expression of several known, direct TR target genes was much higher in the 7-day-old premetamorphic homozygous TRα-knockout tadpoles compared with the wild-type and heterozygous siblings [Fig. 2(b)], suggesting that TRα participated in the repression of these genes during premetamorphosis, when there is little T3.

We next investigated how the knockout animals responded to T3 treatment. We treated 6-day-old, randomly selected tadpoles with 10 nM T3 for 18 hours. Both T3-treated and control tadpoles were euthanized at the same time. The tail tip was clipped for genotyping, and the rest of each animal was homogenized for RNA extraction and expression analysis. As shown in Fig. 3(a), the induction of all direct target genes analyzed was reduced by threefold or more in the homozygous knockout animals compared with the controls. Consistently, when we performed ChIP assays on the T3-treated animals, we observed that homozygous TRα-knockout animals had significantly reduced TR binding to the TREs at endogenous target genes [Fig. 3(b)], indicating that TRα knockout reduced animal response to T3 treatment due to reduced TR binding to target genes, even in the presence of T3. Thus, TRα knockout increases basal expression in premetamorphic tadpoles but reduces the activation of target genes by T3.

**TRα-knockout tadpoles develop faster during premetamorphosis and initiate metamorphosis earlier**

To determine the consequences of the altered expression of T3 target genes in the TRα-knockout tadpoles, we randomly chose tadpoles of the same age and similar size for genotyping and determined their developmental stage, as previously described (51). The results revealed that homozygous TRα-knockout tadpoles were at more advanced stages, as shown by one representative animal for each genotype in Fig. 4(a); the
knockout animal had much more developed hind limbs. To quantify the developmental difference, we randomly selected 55 tadpoles of 11 days of age, and staged (on the basis of limb development) and genotyped each animal. Figure 4(b) shows that, of these 11-day-old tadpoles, the median stage for both the heterozygous and wild-type animals was 47.5, whereas that for the homozygous knockout animals was 51, demonstrating accelerated development of the knockout animals.

Stage 54 is commonly considered to be the onset of metamorphosis, when hind limb morphogenesis/digit formation begins (13, 51). To determine the time required for the animals of different genotypes to initiate metamorphosis, we genotyped tadpoles at 7 days of age and then reared the tadpoles of different genotypes under identical conditions. The age, in days, when each tadpole reached stage 54 was then determined for each genotype and showed that homozygous TRα-knockout tadpoles reached stage 54 in ~20 days after fertilization, whereas the wild-type and TRα heterozygous siblings initiated metamorphosis at the age of ~37 days, nearly twice the age of the homozygous tadpoles [Fig. 4(c)].

Because the initiation of metamorphosis is characterized mainly by the morphogenesis of the limbs, we examined the expression of the hind limb–specific gene Tbx4 and forelimb–specific gene Tbx5 by qRT-PCR analysis of RNA from whole tadpoles at 7 or 15 days of age. In agreement with the accelerated development of the homozygous TRα-knockout tadpoles, we observed that both genes were expressed at significantly higher levels in the homozygous TRα-knockout tadpoles compared with the wild-type and heterozygous siblings [Fig. 4(d)]. Thus, TRα regulates the rate of premetamorphic tadpole development, consequently controlling the timing of metamorphosis initiation.

Removing TRα increases growth rate of premetamorphic tadpoles by upregulating growth hormone gene expression

During our analysis of the developmental stages of the age-matched animals, we noticed that the homozygous TRα-knockout animals appeared to be bigger than the wild-type and heterozygous siblings. To quantify the potential difference, we grouped the 11-day-old tadpoles in Fig. 4(b) into three categories (large, medium, and small) on the basis of their body size [Fig. 5(a–b)]. The results showed that most of the homozygous knockout animals were in the large category, whereas the vast majority of the heterozygous and wild-type animals were in the medium or small category. Given that growth hormone (GH) plays a critical role in regulating growth, and the fact that the
mammalian GH gene is induced by T3 (41, 62), we analyzed the expression of the two GH genes in the tadpoles and observed that both were significantly upregulated in the homozygous TRα-knockout tadpoles [Fig. 5(c)], suggesting that unliganded TRα represses GH expression in premetamorphic tadpoles, and its removal leads to increased GH expression.

To investigate whether the accelerated development was due to the increased growth rate in the homozygous TRα-knockout tadpoles, we next analyzed the size of the animals at the onset of metamorphosis (stage 54), regardless of the age of the tadpoles. Interestingly, we observed that homozygous TRα-knockout tadpoles at stage 54 were much smaller than the wild-type and heterozygous siblings (Fig. 6), and the latter two groups reached stage 54 at much older ages [Fig. 4(c)]. Thus, TRα appears to have two independent functions in premetamorphic tadpoles: controlling the timing of the initiation of metamorphosis and regulating animal growth rate. Upon knocking out TRα, the animals develop so quickly that they reach stage 54 at a much younger age and smaller size, despite their faster growth rate compared with the wild-type siblings during premetamorphosis.

**TRα is required for the normal progression of natural metamorphosis**

As described above, we showed that TRα knockout reduced the response of TR target genes to T3 treatment in premetamorphic tadpoles, which suggests that TRα is important for natural metamorphosis triggered by endogenous T3. To investigate this possibility, we randomly selected tadpoles at stage 54, the onset of metamorphosis, and reared them together. Individual tadpoles were genotyped when they reached stage 58, the beginning of the metamorphic climax. The time required for each animal to develop from stage 54 to stage 58 was recorded and shown in Fig. 7(a). Consistent with the reduced response to T3 treatment shown in Fig. 3, the homozygous TRα-knockout tadpoles took twice as long to reach stage 58 compared with wild-type and heterozygous siblings, indicating that TRα is required for normal progression during the initial phase of metamorphosis.

To determine if the homozygous knockout animals could complete metamorphosis, we let stage 58 animals continue to develop and observed that they were all able to complete metamorphosis, as judged by the total resorption of the tail (not shown). Surprisingly, the time required to progress from stage 58 to stage 66 (the end of metamorphosis) was similar between the homozygous TRα-knockout animals and the wild-type and heterozygous siblings [Fig. 7(b)]. Furthermore, when the age of the individual animals at stage 66 was analyzed, we observed that there were no significant differences among homozygous TRα-knockout animals and heterozygous and wild-type siblings [Fig. 7(c)]. Thus, the early onset of metamorphosis in the TRα-knockout animals was essentially offset by the slow progression of metamorphosis between stages 54 and 58.

**TRα is required for proper coordination of tissuespecific metamorphosis**

It is well known that metamorphosis in different organs is regulated temporally in an organ-dependent
manner. The results described above showed that TRα knockout allowed the animals to initiate metamorphosis sooner, as judged by limb development. On the other hand, tail resorption was completed at similar ages for homozygous TRα-knockout animals and their heterozygous and wild-type siblings [Fig. 7(c)]. Thus, TRα knockout altered the temporal coordination of the tail and limb, two external organs with the most dramatic changes. To investigate if TRα knockout alters the temporal coordination of metamorphosis in other organs, we analyzed the intestine at the end of metamorphosis. During metamorphosis, the intestine is remodeled drastically, which involves the degeneration of larval epithelium and de novo development of the adult epithelium (63–68). One of the most dramatic changes in the intestine is the reduction in its length; the small intestine decreases in length by as much as 90% by the end of metamorphosis (63).

Because homozygous TRα-knockout animals initiate metamorphosis at a younger age and smaller size, we first measured the body size of the animals at the conclusion of metamorphosis and found, not surprisingly, that the knockout animals were significantly smaller at the end of metamorphosis (stage 66) [Fig. 8(a)]. We next measured the length of the intestine for each individual animal at the end of metamorphosis (stage 66). It is well known that animals can differ in size considerably at the end of natural metamorphosis, and that larger animals generally have correspondingly larger organs. Thus, we normalized the length of the intestine against body size. The results showed that the homozygous TRα-knockout animals had significantly longer intestines compared with their wild-type and heterozygous siblings [Fig. 8(b)]. Although our analysis of the intestinal cross-section did not detect significant differences among the three genotypes, the longer length of the intestine suggests that TRα is critical for the complete metamorphosis of the intestine.

To investigate the underlying mechanism for the differential effect of TRα knockout on limb development, intestinal remodeling, and tail resorption, we measured the relative levels of TRα and TRβ mRNAs in the wild-type tadpoles at stage 54, the
onset of metamorphosis, and stage 62 (Fig. 9), the climax of metamorphosis, when T3 level peaks (69). It is interesting to note that at stage 54, limbs had the highest levels of TRα and the highest ratio of TRα to TRβ. This may underlie the largest effect of TRα knockout on limb development. Similarly, TRα expression and the ratio of TRα to TRβ were also much higher in the intestine compared with the tail, which may be the cause of the effect of TRα knockout on intestinal remodeling. Finally, in the tail, TRα expression was very low prior to metamorphosis, and the ratio of TRα to TRβ was the lowest among the three organs, which may explain why TRα knockout has the least effect on the tail. At stage 62, TRβ expression was dramatically upregulated, especially in the intestine and tail, resulting in a much smaller ratio of TRα to TRβ expression. This may be why TRα knockout has little effect at the climax of metamorphosis, as reflected by the similar time needed for all three genotypes to progress from stage 58 to the end of metamorphosis.

**Discussion**

The ability of TRs to bind TRES in chromatin constitutively, but regulate gene expression in a T3-dependent manner, has long implicated a role for both liganded and unliganded TR in normal physiology and pathology. In particular, of the only two known TRs in all vertebrates, TRα and TRβ, TRα is expressed more broadly and prior to the synthesis of endogenous T3 during vertebrate development, implicating a role of unliganded TRα during development. Furthermore, T3 deficiency causes more severe effects on mouse development compared with TR knockout (36–38, 70–73), suggesting that the continued presence of unliganded TRs during development is detrimental to mouse development. However, the potential contribution of maternal T3 to the mouse embryo complicates the interpretation and makes it difficult to ascertain a role for unliganded TR in mammals.

Amphibian metamorphosis is totally dependent on T3 and takes place externally, without maternal influence. This makes it an ideal model to investigate the role of TR during vertebrate development. By using TALEN-mediated knockdown, we and the Buchholz laboratory previously showed that TRα knockout accelerated premetamorphic development and reduced the response to exogenous T3, leading to a reduced rate of metamorphic progression (43–46). On the other hand, the lack of a well-defined, homozygous, total TRα-knockout line in these earlier studies prevented analysis of the role of TRα during natural metamorphosis and left a question about the possible role of the residual TRα in the knockdown animals. Our studies described here, with well-defined TRα–total knockout animals, have not only confirmed these earlier findings but also led to several discoveries. First, TRα...
knockout does not affect embryogenesis. Second, TRα is not essential for amphibian metamorphosis, but it controls the timing and rate of metamorphosis. Finally, TRα knockout has distinct effects on metamorphosis in different organs, enabling limb develop to occur earlier and disrupting the completion of intestinal metamorphosis, but having little effect on tail resorption.

Roles of TRα prior to metamorphosis

Earlier studies have shown that low levels of TRs and T3 are present during *Xenopus* embryogenesis (5, 34, 40, 41, 54–56) and that altering TR levels or function also affects embryonic development (16, 54). However, the role of endogenous TRs in embryogenesis was previously impossible to investigate because of the lack of TR knockout in amphibians prior to the development of gene-editing technologies. TRα is activated earlier and expressed at much higher levels than TRβ during embryogenesis (5, 34, 39–41). Interestingly, our studies have indicated that knocking out TRα has no apparent effect on embryogenesis, suggesting that either the previously reported effects of TR were due to overexpression of wild-type or mutant TR, or that endogenous TRβ was able to compensate for the loss of TRα.

On the other hand, in agreement with the high levels of TRα expression after embryogenesis and throughout premetamorphic development, TRα is critical for tadpole development. First, TRα knockout accelerated premetamorphic development, enabling the animals to initiate metamorphosis earlier. Because T3 levels prior to stage 54 are very low compared with levels during metamorphosis (55, 69), TRα likely functions as unliganded TR to repress target gene expression. Indeed, of the T3-inducible genes that we have analyzed, all were found to be derepressed upon TRα knockout, and accompanied by reduced recruitment of the corepressor NCoR. Thus, our studies provide direct support for the dual-function model for TR during frog development (12, 42). That is, in premetamorphic tadpoles, unliganded TR recruits corepressors to repress target genes to prevent premature metamorphosis, and when T3 becomes available, TR recruits coactivators to activate target gene transcription and initiate metamorphosis.

Unexpectedly, we also observed that TRα-knockout tadpoles grew faster compared with their age-matched wild-type and heterozygous siblings, and that this growth effect might be due to the increased expression of the GH genes. The mammalian GH gene is known to be a T3-inducible gene, and if this regulation is conserved in amphibians, high levels of TRα in premetamorphic tadpoles would repress GH expression, given that T3 levels are very low. Thus, TRα knockout would be expected to cause the derepression of GH expression, resulting in faster tadpole growth.

It is quite reasonable to expect that these two effects of TRα knockout on premetamorphic tadpoles are related to each other. One might expect that faster-growing tadpoles will reach the onset of metamorphosis sooner and thus at the onset of metamorphosis, the animals would be expected to be similar in size. Surprisingly, at the onset of metamorphosis (stage 54), homozygous TRα-knockout tadpoles were much smaller than wild-type or mutant TRs or that endogenous TRα has no apparent effect on embryogenesis, suggesting that either the previously reported effects of TR were due to overexpression of wild-type or mutant TR, or that endogenous TRβ was able to compensate for the loss of TRα.

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![Figure 6. The homozygous TRα-knockout tadpole (Hom) has a smaller body size at the onset of metamorphosis despite a faster growth rate during premetamorphosis. (a) A representative tadpole of each genotype at stage 54, the onset of metamorphosis. Arrows point to the hind limbs. Note that the homozygous TRα-knockout tadpole has a significantly smaller body size but similarly sized hind limbs compared with the heterozygous TRα-knockout (Het) and wild-type (WT) tadpoles at stage 54. (b) TRα-knockout tadpoles have a shorter body length. Animals were allowed to develop to stage 54 regardless of age. The tadpole body length was measured before tail clipping for genotyping. The sample included 15 wild-type, 23 heterozygous TRα-knockout, and 29 homozygous TRα-knockout tadpoles. The body length of individual animals for each genotype was plotted, with the median (darker solid line) for each genotype shown in the figure. Note the median length of the homozygous TRα-knockout tadpoles was about half of that for the heterozygous TRα-knockout and wild-type tadpoles. There was no statistically significant difference between heterozygous TRα-knockout and wild-type tadpoles. Two asterisks (**) indicate a significant difference between the homozygous knockout and other groups (P < 0.01).](https://academic.oup.com/endo/article-abstract/158/6/1985/3069853)
preventing precocious initiation of metamorphosis by repression of other T3-target genes that are critical for metamorphosis.

Roles of TRα during metamorphosis

Given the high levels of TRα mRNA but low levels of TRβ mRNA in premetamorphic tadpoles, it was not surprising that TRα-knockout tadpoles had reduced response to exogenous T3 treatment (Figs. 2–3). Interestingly, although TRα knockout led to reduced binding of TR to endogenous target genes, the remaining TR binding, especially after T3 treatment, was quite high. This appears to be a result of compensation by TRβ. TRβ is a well-known T3-induced gene (41, 58, 74–76), and thus, is repressed by unliganded TR, mainly TRα, in premetamorphic tadpoles, when there is little T3 present. TRα knockout will thus lead to derepression of TRβ (Fig. 2), which will in turn increase the binding of TRβ to TREs in TRα-knockout animals. After T3 treatment of premetamorphic tadpoles, TRβ is further activated by liganded TRβ itself, which further increases TR binding to endogenous TREs to additionally compensate for some of the loss of TR binding that results from the removal of TRα.

The reduced TR function in premetamorphic TRα-knockout tadpoles, as expected, also leads to an inhibition of natural metamorphosis: in the knockout animals, development from the onset of metamorphosis to the beginning of metamorphic climax (stage 58) takes twice as long compared with this stage of development in wildtype and heterozygous siblings, indicating that TRα regulates the rate of metamorphic progression. Interestingly, after stage 58, the rate of metamorphosis among homozygous and heterozygous TRα-knockout and wildtype animals is similar. This is likely a result of the upregulation of TRβ by the metamorphic climax when endogenous T3 levels are high, suggesting that TRβ, which is highly expressed at the climax of metamorphosis, can compensate for the loss of TRα.

When metamorphosis in different organs was analyzed, we also observed that TRα-knockout animals have distinct temporal patterns of metamorphosis in the limb, tail, and intestine compared with heterozygous and wild-type siblings. For the homozygous TRα-knockout animals, metamorphosis proceeds slower than in wildtype and heterozygous siblings. The time required for each animal to reach stage 58 was plotted, with the median (darker solid line) for each genotype shown in the figure. The groups included four wild-type (WT), 10 heterozygous TRα-knockout (Het), and four homozygous TRα-knockout tadpoles. Note that homozygous TRα-knockout tadpoles required over twice as long to reach stage 58 compared with wild-type and heterozygous TRα-knockout tadpoles. Two asterisks (**) indicate a significant difference between the homozygous knockout and other groups (P < 0.05). (b) TRα knockout has no effect at the climax of metamorphosis. The time for each animal to develop from stage 58 to the end of metamorphosis (stage 66) was plotted, with the median (darker solid line) for each genotype shown in the figure. Note that no significant difference was observed among the three genotypes.
animals, limb metamorphosis (stages 54 to 56 for limb morphogenesis, including digit formation) occurs at much younger ages, whereas tail resorption is completed at a similar age compared with wild-type or heterozygous siblings. On the other hand, intestinal remodeling, in particular, the reduction in the length of the intestine, appears to be incomplete or altered at stage 66, which is considered to be the end of metamorphosis, when tail resorption is complete. Thus, TRa is important for the temporal coordination of the metamorphosis of different organs. Furthermore, our analysis of the TRa and TRb expression in different organs suggests that these differential effects are likely a result of the different expression levels of the receptors in these organs during metamorphosis. In addition, T3 availability in different tissues/organs during metamorphosis is also important in regulating the timing and rate of the metamorphosis of different organs/tissues. It would be interesting in the future to determine how TRa knockout affects the expression of various T3 transporters and intracellular binding proteins, and if these factors contribute to tissue-specific metamorphosis.

**Conclusion**

We previously proposed a dual-function model for TR during frog development (12, 42). This model has since been supported by a number of molecular and transgenic studies from different laboratories (8, 10, 16, 19–25, 28, 30, 77–86). A caveat of these studies is that transgenic overexpression may not reflect the role of endogenous factors in development. With the development of TALEN and clustered regularly interspaced short palindromic repeat/associated protein-9 nuclease technologies for editing endogenous genes in *X. laevis* and *X. tropicalis* (31, 32), it is now possible to investigate the role of endogenous genes. By generating TRa–total knockout animals, we have now confirmed the findings from earlier studies involving knockdown animals but, more importantly, made a few unexpected discoveries. First, despite the high levels of TRa expression toward the end of embryogenesis, TRa is not required for embryogenesis. Second, TRa is not essential even for metamorphosis, although it is the predominant TR throughout most of *Xenopus* development. Last, TRa controls the temporal coordination of metamorphosis in different organs.

**Figure 8.** Animals lacking TRa complete metamorphosis with lower body weight but longer intestine. (a) The homozygous TRa-knockout animal (Hom) has a lower body weight at stage 66. Tadpoles were euthanized when they reached stage 66, which is considered to be the end of metamorphosis, when the tail is completely resorbed. Intestine was isolated from each animal, and both intestine and animal body were fixed in a 4% solution of MgSO4, ethylene glycol tetraacetic acid, 3-(N-morpholino)propanesulfonic acid, and formaldehyde overnight at 4°C. After washing in 70% ethanol twice, both body weight and intestinal length were measured. The body weight of each animal was plotted, with the median (darker solid line) for each genotype shown in the figure. Note that the homozygous TRa-knockout animal had significantly lower weight. The group included six wild-type (WT), 11 heterozygous TRa-knockout (Het), and 15 homozygous TRa-knockout animals. The asterisk (*) indicates a significant difference between the homozygous knockout and wild-type groups (P < 0.05). (b) Homozygous TRa-knockout animals had a longer intestine at stage 66. The length of the intestine of animals described in (a) was measured from the bile duct to the large intestine. After normalization against the body weight for each animal, the relative intestine length was plotted with the median (darker solid line) for each genotype shown in the figure.
Without TRα, the animals initiate metamorphosis at younger ages and smaller sizes but are able to complete metamorphosis, as judged by tail resorption, at similar younger ages and smaller sizes but are able to complete metamorphosis at premetamorphosis (stage 54) and climax (stage 62) was determined by real-time PCR and is shown relative to the level in the intestine at stage 62 for (a) TRα and (b) TRβ, or (c) as a ratio of the expression level of TRα to that of TRβ, with the ratio in the intestine at stage 62 set to 1. The region in the box with dashed borders in the upper panel (c) is enlarged and shown in the lower panel. Note that the limb has a much higher level of expression of TRα at stage 54 and higher levels of relative TRα/TRβ expression than the intestine, with the tail having the lowest.

**Figure 9.** Organ-dependent expression of TRα and TRβ genes during metamorphosis. The expression of TRα and TRβ at premetamorphosis (stage 54) and climax (stage 62) was determined by real-time PCR and is shown relative to the level in the intestine at stage 62 for (a) TRα and (b) TRβ, or (c) as a ratio of the expression level of TRα to that of TRβ, with the ratio in the intestine at stage 62 set to 1. The region in the box with dashed borders in the upper panel (c) is enlarged and shown in the lower panel. Note that the limb has a much higher level of expression of TRα at stage 54 and higher levels of relative TRα/TRβ expression than the intestine, with the tail having the lowest.

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