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Regulation of growth rate and developmental timing by *Xenopus* thyroid hormone receptor α

Luan Wen and Yun-Bo Shi*

Section on Molecular Morphogenesis, Program on Cell Regulation and Metabolism, National Institute of Child Health and Human Development, National Institutes of Health, Bldg. 18T, Rm. 106, Bethesda, Maryland 20892, USA

Abstract

Thyroid hormone (TH) is critical for vertebrate postembryonic development, a period around birth in mammals when plasma TH levels are high. Interestingly, TH receptors (TRs), especially TR α , are expressed prior to the synthesis and secretion of zygotic TH, suggesting the existence of unliganded TR during development. However, the role of unliganded TR during mammalian development has been difficult to study, in part due to the relatively weak phenotype of TR knockout mice. Amphibian metamorphosis resembles postembryonic development in mammals and is controlled by TH via TRs. Like in mammals, TR α gene is highly activated and is the major TR expressed prior to the synthesis of endogenous TH. By using TALEN (transcriptional activator like effector nucleases)-mediated gene editing approach, we and others have now shown that unliganded TR α has two independent functions during *Xenopus* premetamorphosis, i.e. inhibiting growth rate and slowing development. Furthermore, molecular and transgenic studies have shown that unliganded TR α accomplishes these via the recruitment of histone deacetylase (HDAC)-containing corepressor complexes to repress the expression of TH-inducible genes.

Keywords

amphibian metamorphosis; de-repression; thyroid hormone receptor; transcriptional regulation; *Xenopus laevis*; *Xenopus tropicalis*

Introduction

Thyroid hormone (TH) is not only important for development and maturation of many organs/tissues in vertebrate but also affects their function in the adult (Hetzel 1989; Lazar 1993; Tata 1993; Atkinson 1994; Freake & Oppenheimer 1995; Silva 1995; Franklyn & Gammage 1996; Shi 1999; Yen 2001). During mammalian development, the plasma levels of TH are high during the period around birth, also referred to as postembryonic development, when many organs such as the brain and intestine mature into the adult form (Hetzel 1989; Tata 1993; Shi 1999; Howdeshell 2002; Ishizuya-Oka & Shi 2011; Hasebe *et al.* 2013).

* Author to whom all correspondence should be addressed., Shi@helix.nih.gov.

The dependence of mammalian embryos on the mother both before birth and during the neonatal period has made it difficult to study how TH signaling affects mammalian development. On the other hand, amphibian metamorphosis, which mimics the postembryonic development in mammals, is totally dependent on TH but independent of maternal influence. This and the ability to easily manipulate this process by controlling the availability of TH to the tadpoles offer an excellent opportunity to study how TH regulates postembryonic vertebrate development.

Thyroid hormone can both regulate gene expression through nuclear TH receptors (TRs) and influence cell behavior through cell surface and cytoplasmic binding protein via the so-called non-genomic pathways (Evans 1988; Lazar 1993; Tsai & O'malley 1994; Davis & Davis 1996; Yen 2001; Buchholz *et al.* 2006). Two highly conserved TR genes, TR α and TR β , exist in all vertebrates (Laudet & Gronemeyer 2002). For TH inducible genes, TRs mainly bind to TH response elements (TREs) as heterodimers formed with 9-cis-retinoic acid receptors (RXRs) in/around the promoters of target genes even in the context of chromatin and repress their expression in the absence of TH. TH-binding to TR leads to chromatin disruption and the activation of these promoters (Wong *et al.* 1995, 1997, 1998; Sachs & Shi 2000; Hsia *et al.* 2001; Hsia & Shi 2002; Buchholz *et al.* 2005; Matsuura *et al.* 2012; Shi *et al.* 2012; Sun *et al.* 2014).

Expression studies have shown that zygotic expression of TR, especially TR α , occurs well before the synthesis and secretion of endogenous TH. In *Xenopus laevis* and *Xenopus tropicalis*, both TR α and RXR α genes are expressed at high levels by stage 45 when tadpole feeding begins while plasma TH is detectable only by stage 54, the onset of metamorphosis (Leloup & Buscaglia 1977; Yaoita & Brown 1990; Wong & Shi 1995; Wang *et al.* 2008). This and other studies have led to a dual function model for TR during frog development. Over the years, a number of laboratories have provided conclusive evidence in support of this model. Here, we will review some of these studies, with a focus on the role of unliganded TR α during premetamorphosis.

Mechanisms of TR function and the dual function model during frog development

Extensive biochemical, molecular, and cell culture studies have shown that TR regulates transcription by recruiting cofactors. For TH-inducible genes, unliganded TR recruits corepressor complexes, such as those containing histone deacetylase HDAC3 and the highly related TR-binding proteins N-CoR and SMRT (Burke & Baniahmad 2000; Glass & Rosenfeld 2000; Guenther *et al.* 2000; Li *et al.* 2000b; Zhang & Lazar 2000; Jones *et al.* 2001; Zhang *et al.* 2002; Ishizuka & Lazar 2003; Jones & Shi 2003; Yoon *et al.* 2003; Shi *et al.* 2012). This leads to local histone deacetylation and transcriptional inhibition (Fig. 1). In the presence of TH, liganded TR releases corepressor complexes and binds coactivator complexes to activate gene transcription. Many TR-coactivators form large complexes that are involved in chromatin disruption/remodeling and/or histone modifications. The best studied among them are SRC (steroid receptor coactivator)-1, 2, and 3, which complex with other proteins including CBP/p300 and protein arginine methyltransferase 1 (PRMT1)

(Onate *et al.* 1995; Wong *et al.* 1995, 1997; Chen *et al.* 1997, 1999; Torchia *et al.* 1997; Li *et al.* 2000a; Zhang & Lazar 2000; Ito & Roeder 2001; Koh *et al.* 2001; McKenna & O'malley 2001; Rachez & Freedman 2001; Sheppard *et al.* 2001; Yen 2001; Demarest *et al.* 2002; Huang *et al.* 2003; Matsuda *et al.* 2007, 2009; Heimeier *et al.* 2008; Matsuura *et al.* 2012; Shi *et al.* 2012).

Like in mammals, there are two TR genes, TR α and TR β , in *Xenopus laevis* and *Xenopus tropicalis* (Yaoita & Brown 1990; Wang *et al.* 2008). There is TR β mRNA expressed during early development, while the expression of TR α and RXR α is activated around hatching (stage 35) and reaches high levels by stage 45 (Fig. 1) (Yaoita & Brown 1990; Kanamori & Brown 1992; Wang *et al.* 2008), the onset of tadpole feeding (Nieuwkoop & Faber 1956). Based on this and the molecular mechanism of TR action, we have previously proposed a dual function model for TR during the frog development (Fig. 1) (Shi *et al.* 1996; Sachs *et al.* 2000). That is, TH-inducible genes are repressed by unliganded TR in the form of TR α / RXR α heterodimers by the end of embryogenesis when a free feeding tadpole is formed at stage 45. This is accomplished through the recruitment of corepressor complexes containing HDACs to ensure proper tadpole growth and prevents premature metamorphic organ transformations, thus regulating the timing of metamorphosis. After stage 54, the availability of endogenously synthesized TH leads the release of the corepressor complexes and concurrent recruitment of the coactivator complexes. The coactivator complexes in turn remodel chromatin and/or modify histones via acetylation and methylation, etc., to activate the genes, leading to tadpole metamorphosis (Fig. 1).

TR is necessary and sufficient for metamorphosis

A prerequisite of the dual function model above is that TR plays an essential role in metamorphosis. Indeed, a number of *in vivo* studies, including transgenic analyses with mutant TRs have provided strong evidence to support this (Puzianowska-Kuznicka *et al.* 1997; Sachs & Shi 2000; Schreiber *et al.* 2001; Das *et al.* 2002; Buchholz *et al.* 2003, 2004, 2005; Nakajima & Yaoita 2003; Schreiber & Brown 2003; Hasebe *et al.* 2011). In particular, overexpression of a dominant negative TR that lacks TH binding ability but retains the ability to heterodimerize with RXR and repress gene expression inhibits not only TH-induced gene activation but more importantly also the metamorphic transformations in all organs/tissues of *Xenopus laevis* tadpoles (Schreiber *et al.* 2001; Das *et al.* 2002; Buchholz *et al.* 2003; Nakajima & Yaoita 2003; Schreiber & Brown 2003). Conversely, premetamorphic tadpoles transgenic for a dominant positive TR under the control of a heat shock-inducible promoter undergo metamorphosis upon heat shock treatment even in the absence of TH (Buchholz *et al.* 2004; Hasebe *et al.* 2011). This is accompanied by the specific activation of TH-inducible genes. Thus, TR is not only necessary but also sufficient to mediate the effects of TH during *Xenopus* metamorphosis.

A role of TR α in regulating both metamorphic rate and timing

A critical test for the dual function model is to demonstrate a role of endogenous TR during *Xenopus* development. While it is possible to knockdown the expression of endogenous genes during metamorphosis by using antisense morpholino oligonucleotides (Matsuda &

Shi 2010), such studies are difficult to carry out and the knockdown is often very limited. The recent development of TALEN and CRISPR-nuclease mediated knockout/knockdown has essentially revolutionized genetic studies of postembryonic development, especially in amphibians where there had been no effective knockout strategy (Lei *et al.* 2012, 2013; Blitz *et al.* 2013; Nakayama *et al.* 2013; Guo *et al.* 2014; Wang *et al.* 2015; Wen *et al.* 2015). Making use of the TALEN technology, we and the Buchholz's laboratory recently investigated the function of endogenous TR α during *Xenopus tropicalis* development (Choi *et al.* 2015; Wen & Shi 2015; Yen 2015). Both groups independently developed a TALEN nuclease targeting TR α and introduced the nucleases into fertilized eggs by microinjecting the corresponding mRNAs. We observed that injecting into fertilized egg prior to the first cell division, mRNAs encoding the two arms of the TR α TALEN nuclease, but not when either TALEN arm was replaced with a non-specific TALEN arm, led to genetic alterations at the TR α target site with about 90% efficiency in the resulting animals, enabling the phenotypic analysis with the resulting F0-generation animals (Wen & Shi 2015). A different approach was taken by the Buchholz's group (Choi *et al.* 2015). They coinjected mRNAs encoding the TALEN nuclease and mCherry into one cell of the two-cell stage embryos. This enabled them to observe the effect of the knockdown in one side of the embryo when compared to the other, wild type side. Both groups observed that TR α knockdown led to precocious limb development (Fig. 2B). The Buchholz's group further confirmed this phenotype by analyzing F1 animals produced from mating two F0 animals generated from the mRNA injection, where they observed that only when both copies of the TR α gene were mutated, were the limbs developed precociously (Choi *et al.* 2015).

Limb morphology is the standard criterion for staging tadpoles between stage 48, shortly after the onset of feeding, and stage 54, the onset of metamorphosis (Nieuwkoop & Faber 1956). By staging the knockdown animals, both group found that TR α knockdown accelerated premetamorphic tadpole development by a few stages (Fig. 2C) (Choi *et al.* 2015; Wen & Shi 2015). Importantly and consistent with the dual function model, the expression of several well known TH-inducible genes were de-repressed (Fig. 2A). Furthermore, the knockdown/knockout animals were resistant to treatment of exogenous TH in terms of both morphological changes and the regulation of TH response genes (Choi *et al.* 2015; Wen & Shi 2015). In addition, when the knockdown animals were allowed to undergo natural metamorphosis, it was found that TR α knockdown drastically slowed down natural metamorphosis as measured by the time required for the animals to advance from stage 54, the onset of metamorphosis, to stage 58, the early metamorphic climax (Wen & Shi 2015). Thus, unliganded TR α indeed plays a critical role in regulating the timing of metamorphosis initiation, while when TH is present, TR α also regulates the rate of metamorphosis progression. This finding also complements earlier studies showing that TR overexpression induces precocious cell death of tadpole tail muscle and thus accelerates metamorphosis (Okada *et al.* 2012).

A novel role of unliganded TR in regulating premetamorphic tadpole growth

A surprising finding of the above studies is that TR α knockdown tadpoles grow faster during premetamorphosis, with the knockdown ones larger when compared to age-matched wild type siblings (Fig. 3A,B) (Wen & Shi 2015). Interestingly, the two growth hormone

(GH) genes are found to be upregulated in the knockdown animals. In mammals, GH is activated at the transcriptional level by TH (Santos *et al.* 1987; Kanamori & Brown 1992). Thus, it is likely that GH is similarly regulated, i.e. repressed by TR in the absence of TH in premetamorphic *Xenopus tropicalis* tadpoles, and TR α knockdown thus leads to de-repression of GH genes, thereby causing faster growth of the premetamorphic tadpoles.

The fact that TR α knockdown causes both faster growth and accelerated development raises the interesting question whether the two effects are dependent. It is reasonable to expect growth and development are tightly related with faster growing animals reaching metamorphosis sooner. In this case, one would expect that the wild type and knockdown tadpoles are similar in size at the onset of metamorphosis (stage 54), although requiring different lengths of time to reach it. Surprisingly, when stage 54 animals are compared, TR α knockdown tadpoles are actually smaller than the wild type siblings (Fig. 3A,B). Thus, the increased growth rate due to TR α knockdown appears to be uncoupled from the acceleration of tadpole development in the knockdown animals. It further suggests that TR α plays a much more important role in slowing development of the animals toward metamorphosis but a minor role in repressing growth (Fig. 4). Thus, even though at the end of embryogenesis and onset of tadpole feeding, the knockdown and wild type animals of the same age are similar in size (Fig. 4), as feeding begins, TR α knockdown animals grow in size faster than wild type siblings and become bigger than the wild type ones when compared at the same age (Fig. 4, at any time point along the horizontal axis prior to reaching stage 54). However, these knockdown animals develop (advancement in developmental stages) much faster, reaching stage 54 at much younger age. The wild type animals grow slower and take longer time to reach stage 54 (i.e. at older age). This gives them extra growth time before initiating metamorphosis (stage 54). The final outcome is that the faster growing TR α knockdown tadpoles are nonetheless smaller at the onset of metamorphosis when compared to the wild type siblings (Fig. 4, the vertical axis value for the animals at stage 54).

A role of HDAC-corepressor complexes in the control of metamorphic timing by unliganded TR

Molecular studies have shown that unliganded TR recruits HDAC containing corepressor complexes, in particular the N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors)-containing HDAC-complexes to target genes. Indeed, chromatin immunoprecipitation (ChIP) analyses have shown that indeed N-CoR and SMRT complexes are recruited by TR in premetamorphic tadpoles to endogenous target genes (Sachs *et al.* 2002; Tomita *et al.* 2004) and importantly, upon TH treatment of premetamorphic tadpoles or during natural metamorphosis, such complexes are released and local histone acetylation levels increase (Sachs *et al.* 2002; Tomita *et al.* 2004), supporting a role of the corepressor complexes and histone deacetylation in the repression of TH target genes and premetamorphic animal development.

To investigate the role of corepressor complexes in premetamorphic tadpoles, we have generated a dominant negative form (dnN-CoR) of the *Xenopus laevis* N-CoR that contained only the receptor interacting domain (ID) near the C-terminus of N-CoR and introduced it

into tadpoles via transgenesis by placing it under the control of a heat shock-inducible promoter (Fig. 5A). Heat shock treatment of the transgenic tadpoles leads to overexpression of the dnN-CoR, which should bind to endogenous TR during the developmental stages 45–54 when TR expression is high while TH levels are low (Fig. 1), a window period of about 30 days (Fig. 5B) (Nieuwkoop & Faber 1956). Thus, it should disrupt the recruitment of endogenous corepressor complexes to the target genes. When transgenic and their wild type siblings were subjected to daily heat shock treatment, starting at stage 46, endogenous TH response genes were indeed derepressed and more importantly, transgenic animals developed faster, reaching stage 54, the onset of metamorphosis, by as much as 7 days earlier than the wild type siblings during the 30 day experiment (Fig. 5C) (Sato *et al.* 2007). Thus, the recruitment of the endogenous HDAC-containing corepressor complexes by unliganded TR is required for gene repression in premetamorphic tadpoles and for proper timing of the initiation of metamorphosis.

Conclusions

Ever since the discovery that TR can bind to TREs both in the presence and absence of TH and can repress gene expression in the absence of TH, it has been suggested that unliganded TR may play a role in vertebrate development. The external development of amphibians offers the best opportunity to test this hypothesis. Indeed, a number of earlier transgenic and molecular studies have provided strong evidence for such a role by unliganded TR during premetamorphosis. Recent knockout/knockdown studies have now provided conclusive evidence that unliganded TR α represses target genes to prevent precocious metamorphosis and that TR α further regulates the rate of metamorphosis when TH is present. Surprisingly, these studies have also revealed a novel function for unliganded TR α . That is, unliganded TR regulates the rate of animal growth prior to metamorphosis in a manner that is uncoupled from its effect on developmental progression toward metamorphosis.

Anuran metamorphosis resembles the postembryonic development in mammal. It is very likely that premetamorphosis in anurans will share similarity with the period of mammalian development prior to the rise of endogenous TH. Indeed, as in amphibians, TR is expressed prior to the maturation of the thyroid gland in mammals, suggesting that similar functions for unliganded TR may exist in mammals. Findings from a number of studies in mammals are consistent with such a view, including the effect of TR α knockout on heart gene expression and development (Mai *et al.* 2004) and the effect of TR β deletion/mutations on the auditory and visual systems (Refetoff *et al.* 1967; Brucker-Davis *et al.* 1996; Forrest *et al.* 1996; Ng *et al.* 2001; Griffith *et al.* 2002; Jones *et al.* 2003). Similarly, deleting the gene encoding the TH-inactivating enzyme, type 3 deiodinase, also causes auditory defects (Ng *et al.* 2009), arguing for the importance of low TH levels (thus leaving more TR unliganded). Finally, mutations in N-CoR and SMRT, two of the best-studied corepressors that bind to unliganded TR, in mice that disrupt corepressor-TR interaction or deacetylase activation lead to derepression of TH-inducible genes, supporting a role of corepressor recruitment by unliganded TR in gene regulation *in vivo* in mammals (Astapova *et al.* 2008; You *et al.* 2010; Pei *et al.* 2011). Thus, it is very likely that unliganded TR may have similar functions during mammalian development, such as coordinating organ growth and development (Choi *et al.* 2015; Wen & Shi 2015; Yen 2015).

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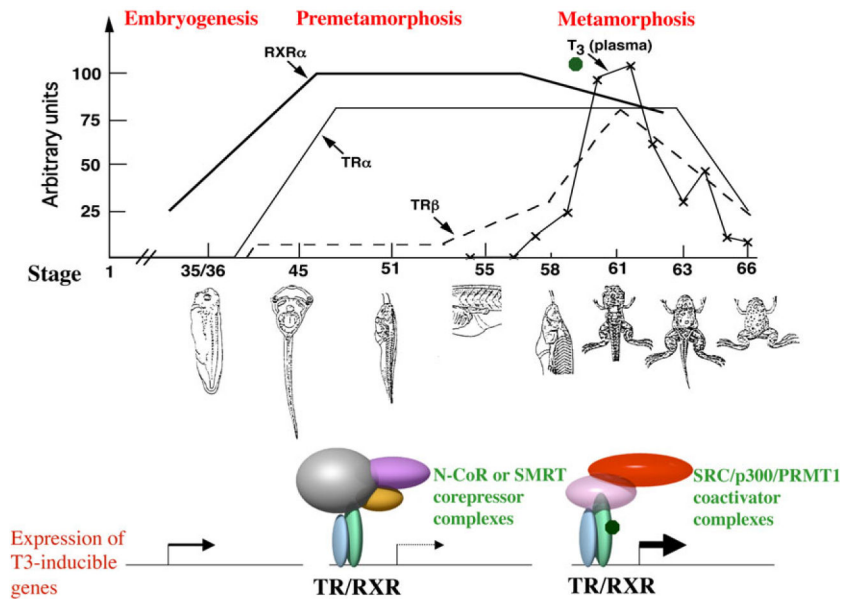


Fig. 1.

A model for the regulation of frog development by thyroid hormone (TH) and TH receptors (TRs). Top: Correlation of the levels of endogenous TH and mRNAs of TR α , TR β and 9-cis-retinoic acid receptor (RXR) α genes with development. The embryos hatch around stage 35 and tadpole feeding begins at around stage 45 (Nieuwkoop & Faber 1956). The plasma concentrations (arbitrary units) of T3 are from (Leloup & Buscaglia 1977) (T3 or 3, 5, 3'-triiodothyronine is one of the two natural THs. The other one T4 or 3, 5, 3', 5'-tetraiodothyronine, also known as thyroxine, binds to TR with weaker affinity). The mRNA levels for TR α (solid line), TR β (broken line) and RXR α (bold line) are based on published data (Yaoita & Brown 1990; Wong & Shi 1995; Wang et al. 2008) and for clarity, plotted on arbitrary scales. Note that there are few morphological changes during premetamorphosis (stage 45–54) when there is little TH in the plasma. Metamorphosis begins after stage 54 (prometamorphosis: stage 54–58) and most dramatic changes occur at climax 58–66). Bottom: A molecular mechanism for the regulation of TH-inducible genes during development. The genes are expressed at the basal levels during embryogenesis when the levels of TH and TRs are low. After tadpole feeding begins at stage 45, TR α and RXR α expression increases dramatically. The resulting TR/RXR heterodimers bind to the TREs in the target genes and recruit corepressor complexes to repress gene expression prior to the synthesis of endogenous TH around stage 54. Subsequently, the rising TH concentration causes the release of the corepressor complexes and recruitment of coactivator complexes to the promoters due to TH binding to TR. This leads to the activation of TH-inducible genes and metamorphosis. Note that there are many different types of corepressor and coactivator complexes that can associate with TR and only exemplary ones are shown here.

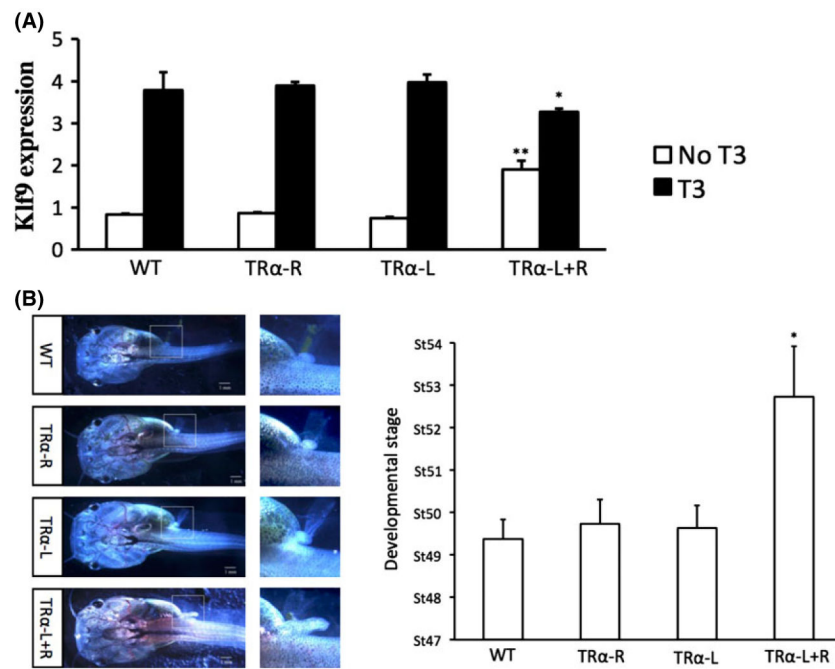
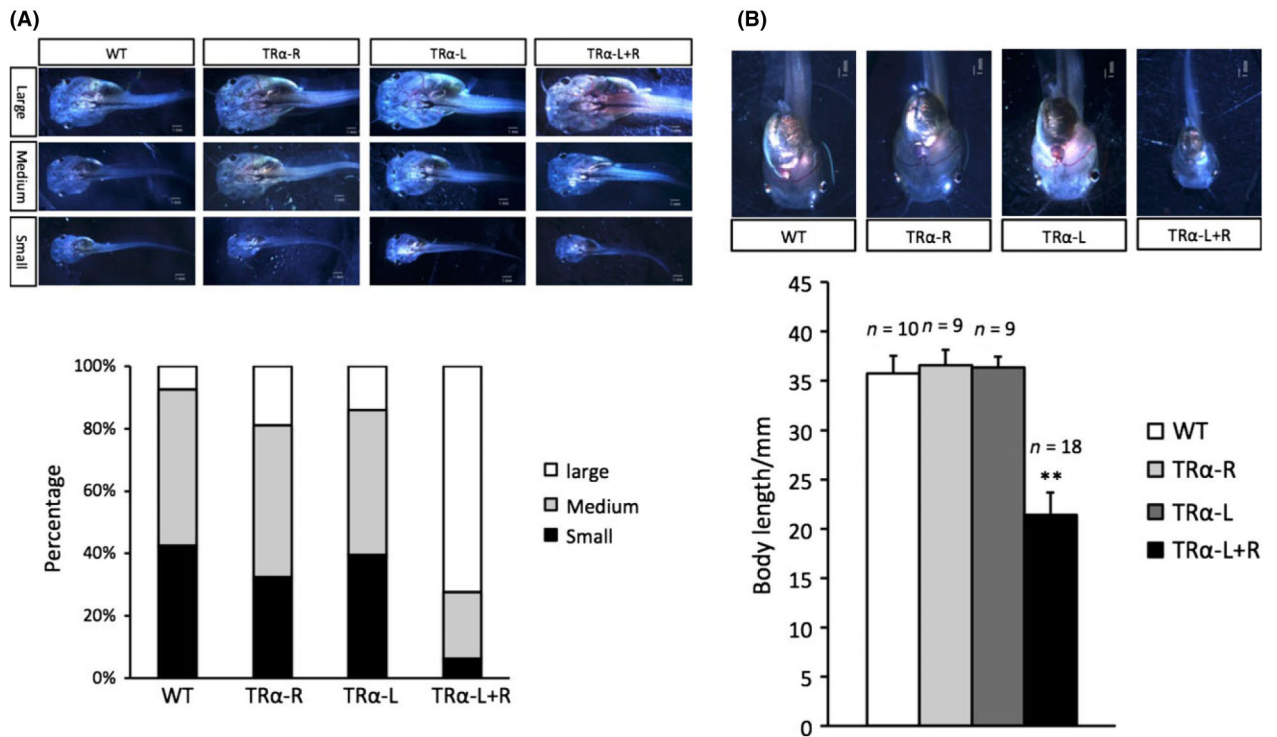


Fig. 2.

(A) Thyroid hormone (TH) receptor α (TR α) knockdown increases the expression of the TH-inducible gene *Klf9* in premetamorphic tadpoles but reduces its induction by TH treatment of *Xenopus tropicalis* tadpoles. TR α was knocked down by injecting mRNAs encoding TALEN left (L) and right (R) arms into fertilized eggs. WT: no TALEN mRNA injection, TR α -R: TR α TALEN-R and control-L mRNAs injected, TR α -L: TR α TALEN-L arm and control-R mRNAs injected, and TR α -L+R: TR α TALEN-L and -R mRNAs injected. Premetamorphic tadpoles were treated with 10 nmol/L TH for 18 h and total RNA was isolated for reverse transcription-polymerase chain reaction (RT-PCR) analysis for the expression of *Klf9*. Note that its expression was higher in the TR α TALEN injected tadpoles in the absence of TH but lower in the presence of TH, respectively, when compared to the WT and control groups, suggesting that both the repression by unliganded TR and activation by TH-bound TR were reduced when TR α was knocked down. ** and * indicate significant difference between the TR α knockdown group compared to all three other groups (** $P < 0.01$, * $P < 0.05$). See (Wen & Shi 2015) for details. (B) TR α knockdown accelerates premetamorphic development. The animals were staged at the age of 20 days post-fertilization. Left panel: Representative animals with the area in dashed box magnified to show the hindlimb size and morphology. Note the more developed hindlimb for the TR α knockdown animal. Right panel shows the average developmental stages of the animals with different TALEN mRNA injections. Note that the TR α TALEN-injected group developed on average 3.5 stages more advanced compared to the other three groups. *Indicates significant difference between the TR α knockdown group compared to all three other groups ($P < 0.05$). See (Wen & Shi 2015) for details.

**Fig. 3.**

(A) Thyroid hormone (TH) receptor α (TR α) knockdown tadpoles have faster growth during premetamorphosis in *Xenopus tropicalis*. Tadpoles with different TALEN mRNA injections as in Figure 2 were classified in three categories: large size, medium size, and small size (top panel), at the age of 20 days post-fertilization and the percentage in each category is shown in a bar graph (bottom panel). (B) TR α knockdown tadpoles are smaller than the wild type siblings at the onset of prometamorphosis (stage 54). Top: Representative photos of stage 54 (the onset of metamorphosis) tadpoles. Tadpoles were staged based on hindlimb morphology. All animals are at the same stage but are of different ages. Bottom: The average size (body length) of the animals at stage 54 for different groups. The letter *n* indicates the number of tadpoles for each group. Tadpole body length was measured from jaw to tail tips. **Indicates significant difference between the TR α knockdown group compared to all three other groups ($P < 0.05$). See Fig. 2 and (Wen & Shi 2015) for details.

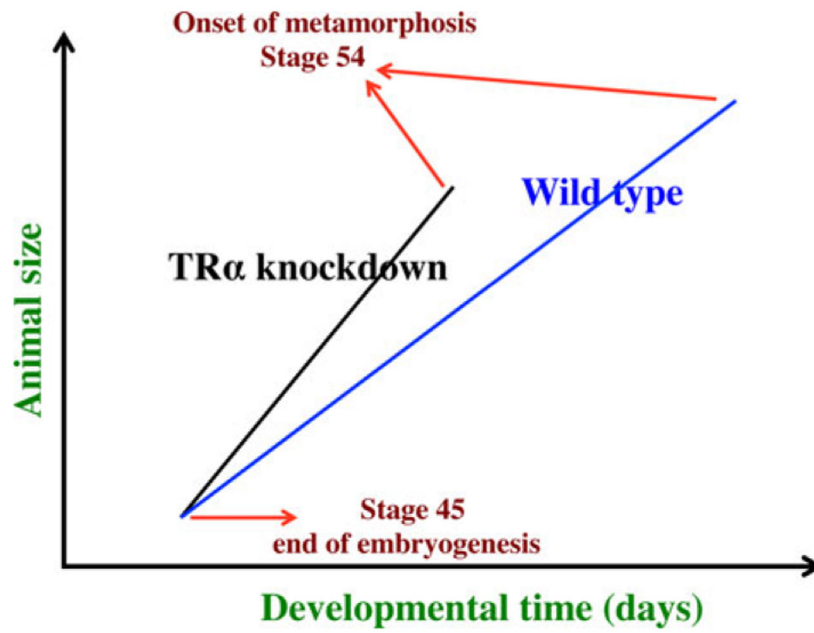


Fig. 4.

Schematics showing the dual effects of thyroid hormone (TH) receptor α (TR α) knockdown on premetamorphic development in *Xenopus tropicalis*. TR α knockdown has little effect on embryogenesis and the resulting tadpoles are normal by feeding stage (stage 45). Once feeding begins, the animals grow differently, with the knockdown ones growing faster, and thus larger when compared to wild type siblings at the same age (in days) (compared with the vertical axis values of the lines for the knockdown and wild type animals at any given position along the horizontal axis between stages 45 and 54). The knockdown animals also have faster development, reaching developmentally more advanced stages when compared to wild type siblings at the same age (in days). Thus, the knockdown animals reach stage 54, the onset of metamorphosis, at a younger age (see the horizontal axis locations for the upper end of the lines). Interestingly, when the animals at stage 54 are compared, the wild type ones are actually larger than the knockdown siblings even though the latter grow faster. This is because the wild type animals take longer to reach metamorphosis (stage 54). The extra growth time needed to reach stage 54 enables the wild type ones to catch up and surpass the knockdown ones in size. These results indicate that the effects of TR on growth and development are not dependent on each other (as otherwise, the size of the animals at stage 54 would be identical between the wild type and knockdown ones).

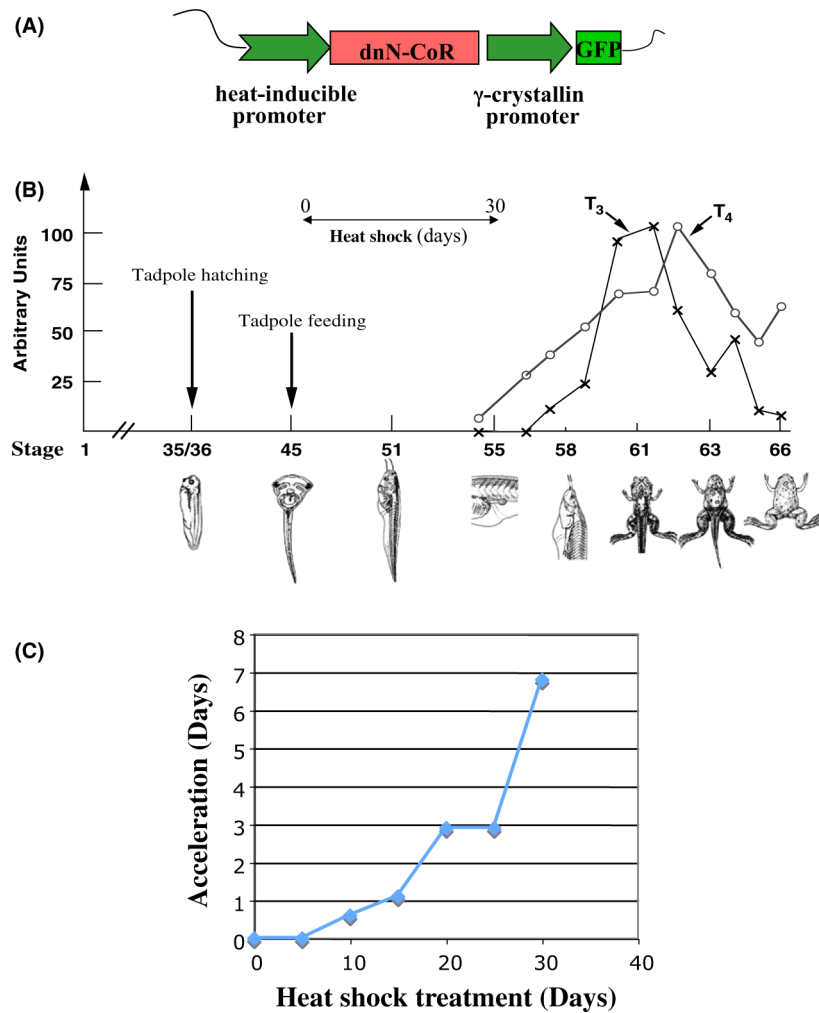


Fig. 5. Transgenic expression of a dominant negative (dn) corepressor leads to premature metamorphosis in *Xenopus laevis*. (A) Schematic representation of dnN-CoR transgenic construct. The dnN-CoR was placed under the control of a heat shock-inducible promoter in a plasmid that also contained the γ -crystallin promoter driving the expression of green fluorescent protein (GFP) for identification of transgenic tadpoles. (B) Experimental strategy. The transgenic and wild type animals are subjected to daily heat shock treatment at the onset of feeding, around stage 45, until they reach stage 54, the onset of metamorphosis when endogenous T₃ and T₄ becomes available (Leloup & Buscaglia 1977), which bind to TR to prevent the binding of dnN-coR to TR. (C) Transgenic tadpoles expressing the dnN-CoR initiate metamorphosis earlier than wild type siblings. Transgenic F1 generation tadpoles and wild type siblings at stage 46 were treated daily with heat shock to induce dnN-CoR expression in the transgenic but not wild type animals and their developmental stages were determined every 5 days for 30 days (note that at the end of the 30-day experiments, transgenic animals had initiated metamorphosis, i.e. passed stage 54, and thus the experiment was ended since the dnN-CoR would have no effect on TR when TH is present). The time (days) needed for the wild type animals to catch up the stage of the transgenic

siblings at different time points of the heat shock treatment was calculated based on the staging by (Nieuwkoop & Faber 1956) and plotted here. See Fig. 1 and (Sato *et al.* 2007) for more details.

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