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Unliganded Thyroid Hormone Receptor Regulates Metamorphic Timing via the Recruitment of Histone Deacetylase Complexes

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Abstract

Anuran metamorphosis involves a complex series of tissue transformations that change an aquatic tadpole to a terrestrial frog and resembles the postembryonic perinatal period in mammals. Thyroid hormone (TH) plays a causative role in amphibian metamorphosis and its effect is mediated by TH receptors (TRs). Molecular analyses during *Xenopus* development have shown that unliganded TR recruits histone deacetylase (HDAC)-containing N-CoR/SMRT complexes and causes histone deacetylation at target genes while liganded TR leads to increased histone acetylations and altered histone methylations at target genes. Transgenic studies involving mutant TR-cofactors have shown that corepressor recruitment by unliganded TR is required to ensure proper timing of the onset of metamorphosis while coactivator levels influence the rate of metamorphic progression. In addition, a number of factors that can influence cellular free TH levels appear to contribute the timing of metamorphic transformations of different organs by regulating the levels of unliganded vs. liganded TR in an organ-specific manner. Thus, the recruitment of HDAC-containing corepressor complexes by unliganded TR likely controls both the timing of the initiation of metamorphosis and the temporal regulation of organ-specific transformations. Similar mechanisms likely mediate TR function in mammals as the maturation of many organs during postembryonic development is dependent upon TH and resembles organ metamorphosis in amphibians.

1. INTRODUCTION

Thyroid hormone (TH) plays critical roles in vertebrate development and adult organ function (Atkinson, 1994; Franklyn & Gammage, 1996; Freake & Oppenheimer, 1995; Hetzel, 1989; Lazar, 1993; Shi, 1999; Silva, 1995; Tata, 1993; Yen, 2001). The most dramatic developmental process controlled by TH is the metamorphosis in anurans, when an aquatic, herbivorous tadpole is transformed into a terrestrial, carnivorous frog (Dodd & Dodd, 1976; Gilbert, Tata, & Atkinson, 1996; Shi, 1999). During anuran metamorphosis, different tissues undergo distinct changes (Dodd & Dodd, 1976; Gilbert et al., 1996; Shi, 1999). Some, like the tail and gills, are completely resorbed. Others, such as the limbs, are formed *de novo*. Most of the organs, however, undergo partial, often drastic remodeling

involving both larval cell death and adult tissue development. For example, in the larval intestine, the major tissue characteristic of the organ, the larval epithelium, degenerates during metamorphosis by undergoing apoptosis (Dodd & Dodd, 1976; Shi, Hasebe, Fu, Fujimoto, & Ishizuya-Oka, 2011; Shi & Ishizuya-Oka, 1996). Concurrently, the adult epithelial stem cells are formed *de novo* through dedifferentiation of some larval epithelial cells through yet unknown mechanisms. These cells then proliferate and differentiate to form the adult intestinal epithelium with a self-renewing system resembling that in the mammalian adult intestine (Ishizuya-Oka & Shi, 2011; Sun & Shi, 2012).

Extensive studies as early as a century ago have firmly established that TH is the causative agent of amphibian metamorphosis (Allen, 1918; Gilbert et al., 1996; Gudernatch, 1912; Shi, 1999). There are two natural THs, T3 (3, 5, 3'-triiodothyronine) and T4 (3, 5, 3',5'-tetraiodothyronine, or thyroxine). T4 is biologically less active and often considered as a pre-hormone, whose deiodination gives to the more active hormone T3. The plasma levels of both T3 and T4 are low or undetectable in embryos or premetamorphic tadpoles and rise to peak at the climax of metamorphosis (Fig. 10.1) (Leloup & Buscaglia, 1977).

Although other vertebrates do not undergo such a complete TH-dependent transformation, TH is known to also play similarly critical roles in their development (Shi, 2013; Tata, 1993). The most obvious and earliest known abnormalities of human body and behavior associated with TH-deficiency are goiter and cretinism (Hetzel, 1989; Tata, 1993). As in amphibians, there is little TH in the embryonic plasma even though the thyroid gland is developed within the first 12 weeks of gestation in human (Hetzel, 1989; Howdeshell, 2002; Tata, 1993). Significant TH production probably does not occur until 20 weeks or later and in the fetal plasma, TH levels remain low until about 4 months prior to birth. The TH levels reach a peak around birth and remain high several months after birth. This post-embryonic/perinatal period in mammals such as human and mouse mimics anuran metamorphosis in many aspects (Atkinson, 1994; Shi, 1999; Tata, 1993). Among them are the transition from aquatic (living in the amniotic fluid) to terrestrial habitat with air-breathing after birth as the lung matures, the switching of the hemoglobin from the fetal to adult types, the increased albumin expression and the induction of urea cycle enzymes in the liver, the maturation of the intestine to the adult form, skin keratinization, and the developmental progression and restructuring of the central and peripheral nervous systems, etc. Thus, it may not be surprising that TH deficiency during neonatal period leads to irreversible, profound neurological deficit, and mental retardation as well as other defects in human (Hetzel, 1989; Hsu & Brent, 1998; Porterfield & Hendrich, 1993). While the importance of TH in mammalian development has long been established, it is difficult to manipulate the uterus-enclosed mammalian embryos for studying the mechanisms of TH action. This contrasts sharply with the total control of anuran metamorphosis by TH and easiness to manipulate this process in tadpoles or organ/primary cell cultures for functional and mechanistic analyses.

Natural metamorphosis in anurans takes place at precise developmental stages, which are defined based on animal morphology, including the presence or absence of the limb or tail or their morphology, etc. (Dodd & Dodd, 1976; Shi, 1999). In *Xenopus*, the onset of metamorphosis is around stage 55 when endogenous TH becomes detectable (Fig. 10.1)

(Dodd & Dodd, 1976; Nieuwkoop & Faber, 1956). Thus, TH synthesis is the primary determinant for the timing of the onset of metamorphosis. TH is synthesized in the thyroid gland as either T3 or T4. In *Xenopus laevis*, the gland first appears in the embryo as a median thickening of the pharyngeal epithelium at the time of tadpole hatching (stages 35/36) (Dodd & Dodd, 1976). This rudiment then develops into a functional larval thyroid gland around stage 53. Subsequently, the plasma TH concentrations increase, reaching peak levels around stage 60, the climax of metamorphosis. Currently, the mechanisms underlying the developmental regulation of the plasma TH levels are unknown. However, as in mammals, many factors appear to be involved. As have been reviewed extensively, these factors include those that influence the growth and maturation of the thyroid gland and hormonal clues that regulate TH synthesis and release from the gland (Denver, 2013; Dodd & Dodd, 1976; Gilbert et al., 1996; Shi, 1999). Regardless of the regulation mechanisms, the synthesis of endogenous TH appears to dictate the timing of metamorphosis initiation.

On the other hand, metamorphosis can be precociously induced in premetamorphic tadpoles as early as at stage 41 for *X. laevis*, only 3 days after fertilization, by the treatment with exogenous TH, while younger tadpoles or embryos are resistant to TH (Shi, 1999; Tata, 1968). Thus, *Xenopus* tadpoles gain competence to respond to TH as early as stage 41, much earlier than the onset of metamorphosis at stage 55. In addition, different organs undergo metamorphosis at distinct developmental stages even though they are exposed to the same circulating plasma TH. Studies over the last two decades have demonstrated a critical role of TH receptor (TR) in both mediating the metamorphic effect of TH and ensuring proper growth and development of different tadpole organs prior to metamorphosis.

2. TR FUNCTION DURING FROG DEVELOPMENT

TH affects development mainly by regulating gene expression through TRs, although non-genomic effects of TH have also been documented (Buchholz, Paul, Fu, & Shi, 2006; Davis & Davis, 1996; Evans, 1988; Lazar, 1993; Tsai & O'Malley, 1994; Yen, 2001). There are two evolutionary conserved TR genes, TR α and TR β , in all vertebrate species and both are members of the nuclear hormone receptor superfamily (Laudet & Gronemeyer, 2002). TRs can bind to TH response elements (TREs) as monomers, homodimers, as well as heterodimers formed with 9-cis-retinoic acid receptors (RXRs), also members of the superfamily. TH can both activate and repress gene expression. TH-inducible genes, TR-RXR heterodimers bind to TREs 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 (Hsia & Shi, 2002; Hsia, Wang, & Shi, 2001; Shi, Matsuura, Fujimoto, Wen, & Fu, 2012; Wong et al., 1998; Wong, Shi, & Wolffe, 1995, 1997).

TR regulates transcription by recruiting cofactor complexes to target genes. In the absence of TH, TR recruits corepressor complexes, such as those containing histone deacetylase histone deacetylase 3 (HDAC3) and the highly related TR-binding proteins N-CoR and SMRT (Burke & Baniahmad, 2000; Glass & Rosenfeld, 2000; Guenther et al., 2000; Ishizuka & Lazar, 2003; Jones, Sachs, Rouse, Wade, & Shi, 2001; Jones & Shi, 2003; Li, Wang, et al., 2000; Shi et al., 2012; Yoon et al., 2003; Zhang, Kalkum, Chait, & Roeder,

2002; Zhang & Lazar, 2000). This leads to local deacetylation of histones H3 and H4 transcriptional inhibition (Fig. 10.1). The binding by TH induces a conformational change in TR, leading to the release of corepressor complexes and the binding of coactivator complexes and gene activation. Among the coactivator complexes include those involved in chromatin disruption/remodeling as well as histone modifications such as those containing the related TR-binding coactivators SRC-1, 2, and 3, which complex with among other proteins CBP/p300 and protein arginine methyltransferase 1 (PRMT1) (Chen et al., 1997, 1999; Demarest et al., 2002; Heimeier, Hsia, & Shi, 2008; Huang, Li, Sachs, Cole, & Wong, 2003; Ito & Roeder, 2001; Koh, Chen, Lee, & Stallcup, 2001; Li, O'Malley, & Wong, 2000; Matsuda, Paul, Choi, Hasebe, & Shi, 2009; Matsuda, Paul, Choi, & Shi, 2007; Matsuura et al., 2012; McKenna & O'Malley, 2001; Onate, Tsai, Tsai, & O'Malley, 1995; Rachez & Freedman, 2001; Sheppard, Harries, Hussain, Bevan, & Heery, 2001; Shi et al., 2012; Torchia et al., 1997; Wong et al., 1995, 1997; Yen, 2001; Zhang & Lazar, 2000).

As the mediators of the effects of TH (see below), TRs and RXRs are expected to be present during amphibian metamorphosis. Indeed, their mRNAs are highly expressed during amphibian metamorphosis. In the pseudo-tetraploid *X. laevis*, there are two TR α and two TR β genes. The TR α genes are activated as early as stage 41, shortly after tadpole hatching at stage 35/36, and reached high levels by stage 45 (Fig. 10.1) (Kanamori & Brown, 1992; Yaoita & Brown, 1990), the onset of tadpole feeding (Nieuwkoop & Faber, 1956), and their expression is maintained at high levels throughout metamorphosis (Fig. 10.1). The TR β genes have little expression in premetamorphic tadpoles but are highly activated during metamorphosis, coinciding with the rise of endogenous TH (Fig. 10.1) (Kanamori & Brown, 1992; Shi, Yaoita, & Brown, 1992; Wang & Brown, 1993; Yaoita & Brown, 1990). Similarly, RXR α and RXR γ are also expressed in premetamorphic as well as metamorphosing tadpoles (Fig. 10.1) (Wong & Shi, 1995). It has been shown that the TR β genes are activated directly by TR via the binding to the promoter region (Ranjan, Wong, & Shi, 1994; Shi et al., 1992), which explains the correlation of the mRNA levels with plasma TH (Fig. 10.1). The mechanisms governing the developmental expression patterns of TR α and RXR genes remain to be determined.

The expression studies led us to propose a dual function model for TR during the frog development (Fig. 10.1) (Sachs et al., 2000; Shi, Wong, Puzianowska-Kuznicka, & Stolor, 1996). During embryogenesis, many TH-inducible genes are expressed at basal levels sufficient for embryonic development. Once organogenesis is completed, that is, by stage 45 when feeding begins (Nieuwkoop & Faber, 1956), these genes are no longer needed. The increased expression of TR and RXR genes (e.g., RXR α , Fig. 10.1) leads to the formation of TR-RXR heterodimers that bind to the TREs of the TH-inducible genes. In the absence of TH, the unliganded TR recruits corepressor complexes to the genes, resulting in the repression of these genes. Since exogenous TH can induce metamorphosis as early as stage 41, the model hypothesizes that this early expression of TR is likely responsible for the tadpole competence to undergo TH-induced metamorphosis. At the same time, gene repression by the unliganded TR ensures proper tadpole growth and prevents premature metamorphic organ transformations, thus regulating the timing of metamorphosis.

After stage 55, the rise of endogenously synthesized TH leads to the binding of TH to the chromatin-bound TR, which releases the corepressor complexes from the target genes with concurrent recruitment of the coactivator complexes. This results in gene activation and metamorphic transformations in the tadpoles.

Over the years, we and others have carried out a series *in vivo* studies, including transgenic analyses with mutant TRs to test this dual function model (Buchholz, Hsia, Fu, & Shi, 2003; Buchholz, Paul, & Shi, 2005; Buchholz, Tomita, Fu, Paul, & Shi, 2004; Das, Schreiber, Huang, & Brown, 2002; Grimaldi, Buisine, Bilesimo, & Sachs, 2013; Hasebe, Buchholz, Shi, & Ishizuya-Oka, 2011; Nakajima & Yaoita, 2003; Puzianowska-Kuznicka, Damjanovski, & Shi, 1997; Sachs & Shi, 2000; Schreiber & Brown, 2003; Schreiber, Das, Huang, Marsh-Armstrong, & Brown, 2001). In particular, overexpression of a dominant negative TR that lacks T3 binding ability but retains the ability to heterodimerize with RXR and repress gene expression just like unliganded TR inhibits TH-induced gene activation and metamorphosis in *X. laevis* (Fig. 10.2) (Buchholz et al., 2003; Das et al., 2002; Nakajima & Yaoita, 2003; Schreiber & Brown, 2003; Schreiber et al., 2001), indicating an essential role of TH-bound TR in gene regulation and metamorphosis. Conversely, overexpression of a dominant positive TR that cannot bind to TH but constitutively activate TH target gene transcription leads to the upregulation of TH response genes and tadpole metamorphosis (Buchholz et al., 2004; Hasebe et al., 2011). These studies have thus demonstrated that TR is not only necessary but also sufficient to mediate the effects of TH during *Xenopus* metamorphosis.

3. UNLIGANDED TR RECRUITS COREPRESSOR COMPLEXES TO CONTROL METAMORPHIC TIMING

The dual function model for TR (Fig. 10.1) predicts that gene repression by unliganded TR during premetamorphosis is important for proper tadpole growth and development before undergoing metamorphic transformations. As summarized above, unliganded TR can recruit corepressor to target promoters to repress their expression. Chromatin immunization (ChIP) analyses have shown that during *Xenopus* development, the TR-binding corepressors N-CoR and SMRT are recruited by TR in premetamorphic tadpoles to endogenous target genes (Sachs et al., 2002; Tomita, Buchholz, & Shi, 2004). These corepressors are released from the target promoters during natural metamorphosis or upon TH treatment of premetamorphic tadpoles. Both N-CoR and SMRT form large complexes containing other proteins such as TBLR1 and HDAC3 (Guenther et al., 2000; Ishizuka & Lazar, 2003; Jones et al., 2001; Jones & Shi, 2003; Li, Wang, et al., 2000; Shi et al., 2012; Yoon et al., 2003; Zhang et al., 2002; Zhang & Lazar, 2000). Consistently, TBLR1 is also recruited by unliganded TR to endogenous target genes in premetamorphic tadpoles and released during metamorphosis, accompanied by local increase in histone acetylation (Sachs et al., 2002; Tomita et al., 2004), suggesting that such corepressor complexes and histone deacetylation are important for the repression of TH target genes and premetamorphic animal development.

To investigate the role of corepressors in premetamorphic tadpoles, we generated two dominant negative forms of the *X. laevis* N-CoR (dnN-CoRs) that contained only the receptor interacting domain (ID) near the C-terminus of N-CoR (Fig. 10.3A). The first dnN-

CoR, myc-ID monomer, comprised just the ID fused to an N-terminal peptide containing the myc tag and a nuclear localizing sequence (NLS). The second dnN-CoR, myc-ID dimer, is similar to myc-ID monomer except with two copies of the ID in the same directions connected by a linker sequence. In the reconstituted frog oocyte transcription system where one can study transcription of a reporter gene in the context of chromatin, both dnN-CoRs were able to compete against wild type N-CoR for binding to unliganded TR (Sato et al., 2007). More importantly, they reduced the repression of the TH target promoter by TR in the absence of TH with the myc-ID dimer more effective, while having no effect on the activation by TR in the presence of TH (Fig. 10.3B) (Sato et al., 2007).

To investigate the role of corepressor complex in TR function during development, we carried out transgenesis to express the myc-ID under the control of a heat shock-inducible promoter. In the absence of heat shock, the dnN-CoR was not expressed and the transgenic tadpoles and frogs were apparently normal. As this dominant N-CoR binds only to unliganded TR, it is expected to affect TR function only during the developmental stages 45–55 when TR expression is high while TH levels are low (Fig. 10.1), a window period of about 30 days (Nieuwkoop & Faber, 1956). Thus, we subjected transgenic and their wild type siblings to daily heat shock treatment, starting at stage 46. Heat shock treatment led to overexpression of the dnN-CoR and upregulation of most of the TH-response genes analyzed (Sato et al., 2007). More importantly, transgenic animals developed faster, reaching stage 55, the onset of metamorphosis, by as much as 7 days earlier than the wild type siblings within the 30-day experiment (Sato et al., 2007) (Fig. 10.3C). These observations demonstrated that the recruitment of HDAC-containing corepressor complexes is indeed important for gene repression by unliganded TR in premetamorphic tadpoles as the dual function model predicted, and more importantly, plays a role in regulating the timing for the initiation of metamorphosis, thereby ensuring proper growth of the tadpole before its transformation into a frog, an important measure for the growth and survival of the postmetamorphic animal.

4. COACTIVATOR RECRUITMENT FOR METAMORPHIC PROGRESSION

Transcriptional activation by TH involves both the relief of the repression caused by unliganded TR and additional activation due to liganded TR. Expression and ChIP analyses have shown that a number of coactivators, including histone acetyltransferases SRC3 and p300 and histone methyltransferase PRMT1, are expressed and/or recruited by TR to endogenous target genes during *X. laevis* metamorphosis (Havis, Sachs, & Demeneix, 2003; Matsuda et al., 2009, 2007; Paul, Buchholz, Fu, & Shi, 2005, 2007; Paul, Fu, Buchholz, & Shi, 2005; Paul & Shi, 2003). More importantly, transgenic overexpression of a dominant negative form of *Xenopus* SRC3 that contains only the TR-ID (Paul, Fu, et al., 2005), or a dominant negative form of p300 that contains only the SRC-binding domain and is expected to bind to SRCs to disrupt the function of SRC-p300/CBP complexes (Paul et al., 2007), inhibits TH-induced gene activation and TH-metamorphosis as well as natural metamorphosis. These results demonstrate an essential role of the recruitment of coactivator complexes, in particular those containing SRC-p300/CBP, in gene regulation by TR and metamorphosis.

Interestingly, when we overexpressed the full length *X. laevis* PRMT1, another component of the SRC-p300 complexes, in transgenic tadpoles under the control of a heat shock-inducible promoter, heat shock treatment led to increased levels of PRMT1 in tadpoles. The resulting tadpoles had enhanced target gene expression in the presence of TH and accelerated natural as well as TH-induced metamorphosis (Fig. 10.4) (Matsuda et al., 2009). Thus, coactivator levels appear to also influence the rate of the progression of metamorphosis.

5. TEMPORAL REGULATION OF ORGAN-SPECIFIC METAMORPHOSIS

Aside from metamorphosis initiation, the timing of metamorphosis is also reflected at the level of individual organs as different organs undergo their unique metamorphic changes at specific developmental stages. For example, in *Xenopus*, the hindlimb bud begins to grow around stage 48 and the most dramatic morphological differentiation takes place around stages 53–58 (Fig. 10.5). Subsequently, the hindlimb undergoes mostly growth with little morphological changes (Fig. 10.5) (Nieuwkoop & Faber, 1956). On the other hand, tail metamorphosis, that is, total resorption, is one of the last changes to occur. Although some changes in the tail, such as fin resorption, take place earlier, the reduction in tail length does not occur until around stage 62 and its completion marks the end of metamorphosis (Fig. 10.5) (Dodd & Dodd, 1976). Finally, the intestine begins its remodeling around stage 58 when its connective tissue and muscle layers increase in thickness (Ishizuya-Oka & Shimozawa, 1987; McAvoy & Dixon, 1977; Shi & Ishizuya-Oka, 1996). This is followed by the degeneration of the larval epithelium through programmed cell death (apoptosis) around stages 60–62 and the formation of the adult epithelium toward the end of metamorphosis (Fig. 10.5). A number of factors appear to contribute to this temporal regulation of organ-specific metamorphosis. These factors affect two aspects of TH signaling: TR-RXR heterodimer levels and availability of intracellular TH.

Expression analyses of TR and RXR have shown that the mRNAs for TR α , TR β , and RXRs are highly expressed during amphibian metamorphosis (Davey, Schneider, & Galton, 1994; Eliceiri & Brown, 1994; Fairclough & Tata, 1997; Helbing, Gergely, & Atkinson, 1992; Kawahara, Baker, & Tata, 1991; Schneider & Galton, 1991; Wang & Brown, 1993; Wang, Matsuda, & Shi, 2008; Wong & Shi, 1995; Yaoita & Brown, 1990). Importantly, the expression of TR and RXR genes in individual organs during *Xenopus* metamorphosis strongly correlates with the temporal regulation of organ-specific transformations (Eliceiri & Brown, 1994; Wang & Brown, 1993; Wang et al., 2008; Wong & Shi, 1995; Yaoita & Brown, 1990). In general, TR and RXR genes are coordinately regulated during metamorphosis and their expression is high in a given organ when metamorphosis takes place and low before or after metamorphosis (Table 10.1). Thus, the mRNA levels for these receptor genes (except for TR β genes) are high in the hindlimb around stages 54–56 when hindlimb morphogenesis, for example, digit formation, takes place (Wang & Brown, 1993; Wang et al., 2008; Wong & Shi, 1995). Similarly, in the tail, the genes are expressed at low levels until stage 62 when they are up-regulated, coinciding with the rapid tail resorption, while in the intestine, high levels of TR and RXR mRNAs are present during tissue remodeling (stages 58–66) (Table 10.1) (Wang & Brown, 1993; Wong & Shi, 1995). Thus,

the levels of TR-RXR heterodimers likely help to determine temporal regulation of the changes in individual organs.

In the absence of TH, the presence of TR-RXR in a given organ can act to prevent precocious metamorphosis of the organ, thus ensuring its function in the developing tadpoles. TH levels in an organ is affected several mechanisms. The first is the circulating plasma levels of TH, governed mainly by the production of TH in the thyroid gland (Table 10.1 and Fig. 10.1) (Leloup & Buscaglia, 1977). Secondly, the levels of cellular TH could be affected by cellular proteins that bind TH, that is, cytosolic thyroid hormone binding proteins (CTHBPs). Finally, the metabolic conversion of T₄ to T₃ and/or the inactivation of both hormones may be an important step in regulating the levels of functional TH within an organ.

While all organs are exposed to the same circulating plasma TH, once TH enters the cytoplasm, it is likely to CTHBPs (Ashizawa & Chen, 1992; Barsano & Groot, 1983; Shi, Liang, Parkison, & Cheng, 1994; Yamauchi & Tata, 1994). In general, CTHBPs are multi-functional proteins that serve one or more other roles (e.g., pyruvate kinase, myosin light chain kinase, and disulfide isomerase) and bind TH with 10–100-fold weaker affinity than the nuclear TRs. The exact roles of these CTHBPs in TH signal transduction are still unclear. These proteins could participate in TH import from the extracellular medium, intracellular TH metabolism, and transport to the nucleus, or serve as a buffer to modulate intracellular free TH concentrations. Studies on the human M2 pyruvate kinase (PKM2) have shown that over-expression of the protein leads to an inhibition of TH-dependent transcriptional activation by TR in a tissue culture cell line (Ashizawa & Chen, 1992). Interestingly, analysis of the expression of the frog PKM2 in the tail, limb, and intestine revealed that the intestine had very low but relatively constant levels of its mRNA during development (Shi et al., 1994). In contrast, the PKM2 expression in the tail and hindlimb was drastically altered during metamorphosis. Low levels of PKM2 mRNA were present in either the hindlimb during morphogenesis (stages 54–56) or tail during resorption (stages 62–64) (Table 10.1). Interestingly, PKM2 mRNA levels increased dramatically in the growing hindlimb and decreased precipitously in the resorbing tail. Thus, while recent studies suggest that PKM2 may affect cell fate via multiple mechanisms (Lu, 2012; Luo & Semenza, 2012), these findings support a potential role of PKM2 in the regulation of free cellular TH to affect the metamorphic transition of these two organs.

Another factor affecting local TH levels is deiodination. TH is synthesized initially as T₄ or thyroxine in the thyroid gland (St Germain et al., 1994). Some T₄ in turn is converted in the thyroid gland into T₃ by 5'-deiodinases (Jorgensen, 1978; St Germain et al., 1994). Both T₃ and T₄ are then secreted into the serum and carried to target tissues (Hennemann & Visser, 1997; Oppenheimer et al., 1987; Ritchie et al., 2003; Robbins, 1992; Shi, Ritchie, & Taylor, 2002; Visser, Friesema, & Visser, 2011). There are two families of deiodinases, 5'- and 5-deiodinases (Arrojo & Bianco, 2011; Gereben et al., 2008; St Germain et al., 1994). The 5'-deiodinases convert T₄ to T₃ and thus enhance TH signaling while the 5-deiodinases inactivate both T₄ and T₃, thus reducing TH signaling. Analysis of the expression of the 5-deiodinase gene in *X. laevis* showed that it was regulated in an organ-specific manner (Table 10.1) (St Germain et al., 1994; Wang & Brown, 1993). It had little expression in the

intestine. In the hindlimb, it is not expressed until after limb morphogenesis is completed, that is, around stage 60, when its mRNA level is up-regulated, paralleling the increase in limb size. In contrast, relatively high levels of its mRNA are present in the tail during premetamorphic stages. Immediately prior to rapid tail resorption (stages 58–61), the expression of the 5-deiodinase gene is suddenly up-regulated several fold. Its mRNA levels then return to the premetamorphic levels as tail resorption takes place after stage 61. While no detailed analyses have been done on 5'-deiodinase during *X. laevis* metamorphosis, studies in *Rana catesbeiana* argue that 5'-deiodinase expression is also temporally regulated in different organs such that the enzyme likely functions to increase T3 level within the organ as metamorphosis begins (Becker, Stephens, Davey, Schneider, & Galton, 1997; Davey, Becker, Schneider, St Germain, & Galton, 1995). These findings suggest that 5 and 5'-deiodinases function to regulate the levels of TH within an organ to regulate its metamorphic transformation.

While there are clearly many other factors contributing to the regulation of TH levels within an organ, the brief summary above suggests that the levels of both receptor and cellular free TH in individual organs during metamorphosis contribute to the timing of tissue-specific metamorphosis. At the mechanistic level, low levels of TH in a given organ will lead to a high fraction of TR-RXR heterodimers in the unliganded state. This will lead to the recruitment of HDAC-containing corepressor complexes to the TH target genes to repress their expression and thus prevent organ metamorphosis. Thus, there is likely a tissue-specific critical ratio of liganded to unliganded TR that determines the timing of organ metamorphosis.

6. CONCLUSIONS

Anuran metamorphosis is one of the most dramatic events regulated by TH. It is tightly controlled during development both on the timing of the initiation of this process at whole animal level and on the temporal coordination of organ-specific transformations. The biosynthesis of endogenous TH and the regulation of cellular free TH levels are the key determinants controlling the timing of metamorphosis. Mechanistically, the metamorphic effect of TH is mediated by TR through regulating the transcription of TH-target genes. Low levels of TH ensure a high fraction of TR in the unliganded state at the target genes. This leads to the recruitment of HDAC-containing corepressor complexes to repress the genes, thus preventing the onset of animal metamorphosis and organ transformations. During metamorphosis, high levels of TH lead to liganded TR that activates target gene transcription to induce metamorphosis. Such a ligand switch behavior controls the impressive morphological development of the frog.

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Figure 10.1.

A dual function model of TR in frog development. Top: Correlation of the levels of endogenous TH and the mRNAs of TR α , TR β , and RXR α genes with development in *X. laevis*. The embryos hatch around stage 35 and tadpole feeding begins around stage 45 (Nieuwkoop & Faber, 1956). The plasma concentrations (arbitrary units) of T₃ (crosses) and T₄ (open circles) are from Leloup and Buscaglia (1977). The mRNA levels for TR α (solid line), TR β (broken line), and RXR α (bold line) are based on published data (Wong & Shi, 1995; Yaoita & Brown, 1990) and for clarity, plotted on arbitrary scales. Bottom: Regulation of TH-inducible genes during frog development. TH-inducible 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. In the absence of the TH, the resulting TR-RXR heterodimers bind to the TREs in the target genes and recruit corepressor complexes to repress gene expression. After stage 55, the rise of endogenous TH concentration causes the release of the corepressor complexes and recruitment of coactivator complexes to the promoters due to its binding to TR. This leads to the activation of TH-inducible genes and metamorphosis.

dnTR expression

Morphological
changes

TH response
gene expression

Figure 10.2.

Dose-dependent inhibition of T3-induced metamorphosis by the expression of a dnTR.

Transgenic tadpoles were generated to overexpress a dnTR with GFP fused at the N terminus. The premetamorphic tadpoles were treated with T3 to induce metamorphosis.

Note that the degree of inhibition of T3-induced metamorphosis correlates with the level of dnTR overexpression as judged from the GFP expression levels in the tadpoles (DR Buchholz and Y-B Shi, unpublished). See Buchholz et al. (2003) for more details.

Figure 10.3.

Transgenic expression of a dominant negative corepressor leads to premature metamorphosis. (A) Schematic representation of dnN-CoRs. *X. laevis* N-CoR interacts with unliganded TR via the receptor ID near the C-terminus. The repressor interacting domains (RDs) located in the N-terminal part are required for the recruitment of other co-repressors such as TBL1/TBLR1 and HDAC3. One dnN-CoRs, myc-ID monomer, comprises the ID (amino acids 1988–2349) fused to an N-terminal peptide containing a myc tag and a NLS. The other dnN-CoR, myc-ID dimer, consists of two IDs in the same directions connected by a linker sequence. (B) Of the two dnN-CoRs, myc-ID dimer relieves reporter gene repression by unliganded TR more efficiently than myc-ID monomer. The mRNAs for FLAG-tagged TR and RXR with or without the mRNA for myc-ID dimer or monomer were injected into *X. laevis* oocyte cytoplasm. The TRE-containing firefly luciferase reporter vector together with the control Renilla luciferase plasmid was then injected into the nucleus. After overnight incubation with or without T3, the oocytes were lysed and assayed for luciferase activities. The ratio of firefly luciferase activity to Renilla luciferase activity was determined as a measure of the reporter gene transcription level. The result was expressed as a percentage of the basal transcription level that was obtained from the oocytes without TR/RXR mRNA injection. Note that both dnN-CoRs relieved the unliganded TR-induced repression, but the derepression was more potent with the myc-ID dimer (left graph,

* $p < 0.05$). Neither of dnN-CoRs affected the liganded-TR induced reporter gene activation (right graph). (C) Transgenic (Tg) tadpoles expressing the dnN-CoR myc-ID dimer under the control of a heat shock-inducible promoter initiate metamorphosis earlier than wild type (Wt) siblings. Wild type and transgenic sibling animals at stage 46 were treated daily with heat shock every day to induce dnN-CoR expression and their developmental stages were determined every 5 days for 30 days. Note that at the end of the 30-day experiments, Tg animals had initiated metamorphosis (passed stage 55) while Wt ones were one stage behind (a delay of about 7 days). * p values < 0.05 (Tg vs. Wt). See for Sato, Buchholz, Paul, and Shi (2007) more details.

Figure 10.4.

Transgenic expression of PRMT1 accelerates metamorphosis. (A) Over-expression of PRMT1 accelerates natural metamorphosis. A wild type (Wt) and transgenic (Tg) tadpole overexpression PRMT1 under a heat shock-inducible promoter control developed to stages 60 and 61 (note the pointier head of the transgenic animal, arrow), respectively, after 6 days of heat shock treatment starting at stage 58, the onset of metamorphic climax. (B) PRMT1 overexpression also leads to faster development during T₃-induced metamorphosis. Wt and Tg animals were heat-shocked daily starting at stage 56. Three days after the first heat-shock treatment, all the animals were treated with T₃ and continued to be subjected to heat shock treatment daily. The average developmental stages for the Wt and Tg animals at indicated time points were plotted. **p* values <0.05 when compared to the Wt animals. See Matsuda et al. (2009) for more details.

Figure 10.5.

Temporal regulation of tissue specific metamorphosis. The developmental stages are based on Nieuwkoop and Faber (1956). The tails at stages 62–66 are drawn to the same scale to show the resorption (no tail at stage 66), while the tadpoles, intestinal cross-sections and hindlimbs at different stages are drawn in arbitrary sizes to highlight the morphological differences. Tadpole small intestine has a single epithelial fold, where connective tissue (CT) is abundant, while a frog has a multiply folded intestinal epithelium (EP), with elaborate connective tissue and muscle (MU). Dots, proliferating adult intestinal epithelial cells; open circles, apoptotic primary or larval intestinal epithelial cells; L, intestinal lumen.

Correlations of the relative expression levels of TR, RXR, and some factors affecting cellular free T3 with organ transformations during *Xenopus* metamorphosis.

Table 10.1

Stages	Intestine	Hindlimb	Tail
	58-66	54-56	58-66 54-60 62-66
Plasma T ₃	High	Low	High Low-high High
CTHBP/PKM2 mPvNA	Low-moderate	Low	Increasing High Low
5-Deiodinase mRNA	Low	Low	Increasing Increasing Decreasing
Estimated free cellular T ₃	High	Moderate	Low Low High
TR, RXR mRNA	Moderate-high	Very high	Low Increasing High
Developmental event	Remodeling	Morphogenesis	Growth Few changes Resorption