



## **as a model for studying thyroid hormone signalling: From development to metamorphosis**

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***Xenopus laevis* as a model for studying thyroid hormone signalling: from development to metamorphosis.**

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**Summary:**

Amphibian metamorphosis is a well-established model for dissecting the mechanisms underlying thyroid hormone (TH) action. How the prohormone,  $T_4$ , the active form,  $T_3$ , the deiodinases and the nuclear receptors (TRs) contribute to metamorphosis in *Xenopus* has been extensively investigated. Our recent work has concentrated on two key ideas in TH signalling in *Xenopus*: first, that there could be active roles for both liganded and unliganded receptors, and second, that ligand availability is a determining factor orchestrating these actions and is tightly controlled in target tissues. Recently, we addressed these questions at stages preceding metamorphosis, i.e. during embryogenesis, before differentiation of a functional thyroid gland. We show that repression by unliganded TR is essential to craniofacial and eye development during early development and that at these stages all three deiodinases are active. These results open new perspectives on the potential roles of TH signalling during embryogenesis.

**Introduction: *Xenopus leavis* and thyroid hormone signalling**

Amphibian development is indirect. Embryogenesis and adult development are separated by a larval period (corresponding to tadpole growth) that ends with metamorphosis. Although complex, metamorphosis is initiated and orchestrated by one signal, thyroid hormone (TH). In 1912, Friedrich Gudernatsch fed tadpoles several horse organs and found that feeding the thyroid gland induced metamorphosis. Shortly after, the active substance was identified as thyroxine,  $T_4$  (Kendall, 1915). Later studies led to the structural characterization of the two natural TH (Gross and Pitt-Rivers, 1952; Roche et al., 1952a; Roche et al., 1952b): 3, 5, 3', 5'-tetraiodothyronine ( $T_4$ ) and 3, 5, 3'-triiodothyronine ( $T_3$ ). J.R. Tata made the first key observations on TH action and frog development when he showed that TH induced messenger

RNA (mRNA) synthesis (Tata, 1963; 1966; 1974). A nuclear mode of action was proposed, but it was not until 1986 that mammalian TRs were cloned, followed by the *Xenopus* TRs in 1990 (Yaoita and Brown, 1990). TRs are transcription factors that regulate gene expression by binding to DNA at specific sites qualified as response elements (T3RE; for review see Mangelsdorf et al., (1995); Mangelsdorf and Evans, (1995)). TH is a versatile player.  $T_3$ , through TRs, not only upregulates, but also downregulates, gene expression in target cells (Lezoualc'h et al 1992; Feng et al., 2000). The majority of  $T_3$ -response genes are upregulated by TH and most of the studies on TR mechanisms of action have analysed such genes. Interestingly, *in vitro* and *ex vivo* experiments have revealed that in the absence of  $T_3$ , TRs repress transcription of positively regulated genes (Damm et al., 1989). Over the last decade or so it has been shown that this repression is due to recruitment of co-repressor complexes (Hörlein et al., 1995; Chen and Evans, 1995). Thus, unliganded TRs can work as active repressors (Fondel et al., 1993). Moreover, liganded and unliganded TR are distinct entities with different functions. The physiological role of repression by unliganded TR is not clear. Using the amphibian development model, we have analyzed the potential role of unliganded TRs and the molecular mechanism involved in this repression, particularly during the early developmental stages before the metamorphic program is induced. As repression by TRs implies lack of ligand it is of interest to examine the presence first, of TH and second, of the deiodinases in those tissues that express the receptors. Recent work from our laboratory shows that each of these components of TH signalling, ligand, deiodinases and receptors are expressed in tissue specific manners in early development. Here, we synthesis the recent data and argue that cell and tissue specific responses involving TRs are found during early development. Most notably, eye and craniofacial development requires repression through ligand-independent TR action. This review thus addresses in turn, first the data on active repression by ligand-free TRs and second, data showing that ligand availability is tightly controlled and limited by early expression of deiodinases.

## I. Control of ligand availability by activating and inactivating deiodinases

TH signalling implicates a series of major actors. First, the pro-hormone 5', 3', 3, 5 Tetra-iodo-thyronine ( $T_4$ ) enters the cell via transporters, such as MCT8 (Friesema et al., 2003; Trajkovic et al., 2007). In the target cell,  $T_4$  is converted into the active form 3', 3, 5 tri-iodo-thyronine ( $T_3$ ) by highly specialized enzymes, *ie* the deiodinases. Deiodinases are selenoproteins that catalyse the deiodination of TH at specific sites (Fig. 1). Three deiodinases have been characterised in different vertebrates (D1, D2 and D3, see Galton (2005) for review). Until recently, only two had been characterized in *Xenopus*, the deiodinases D2 and D3: A number of studies clearly demonstrate the vital and specific roles of D2 and D3 in controlling tissue availability of the receptor-active TH,  $T_3$ , during metamorphosis (Huang et al., 2001; Cai and Brown, 2004). Turning to the D1 enzyme, it was first identified in mammals and birds (Berry and Larsen, 1993; Van der Geyten et al., 1997), and more recently in fish (Sanders et al., 1997; Orozco et al., 2003). D1 carries out both ORD and IRD reactions and thus can be seen as either an activating or inactivating deiodinase. Expression of type 2 deiodinase in *Xenopus* has been shown to correlate with the beginning of metamorphosis, when it is first expressed in developing limb buds. In particular, D2 has been shown to be involved in setting up the negative feedback loop exerted by TH on pituitary function, with a delay in expression until the high circulating levels of TH are reached during metamorphic climax. Moreover, D2 expression is concomitant with the major tissue changes occurring during metamorphosis (including tail resorption, intestine and brain remodeling and limb development). Over expression of D3 in *Xenopus laevis* protects tissues from induced metamorphosis and blocks natural metamorphosis of certain tissues such as the tail. D3 activity is differentially regulated during metamorphosis, in certain cases, such as in the tail, being down-regulated as D2 is up-regulated. D3 also determines differential availability of active TH in the retina. Higher, constitutive, expression of D3 in the dorsal ciliary margin limits  $T_3$  availability, resulting in asymmetric replication of the ventral retina. Further,

overexpression of D3 in germinally transgenic tadpoles inhibits certain metamorphic changes such as tail resorption. Thus TH availability in cells is tightly regulated by the balance between the activity of the three deiodinases.

In mammals and birds, D1 is sensitive to inhibition by propylthiouracil (PTU). However, in amphibians PTU treatment is without effect on deiodinase activity. Such findings led to the assumption that D1 did not exist in amphibians. However, recently we demonstrated the presence of a functional D1 in *Xenopus*. When investigating the expression pattern of this newly characterized deiodinase we found it was expressed early in development and thus investigated the patterns of expression of each deiodinase during embryogenesis.

### **1.1. Both activating and inactivating deiodinases are present in early *Xenopus* embryos**

The timing of expression of each deiodinase gene during embryogenesis was determined by QPCR. Detectable levels of mRNA encoding the three deiodinases were found in *Xenopus laevis* embryos from neurulation stages onwards. More precisely, in *X. laevis* eggs and whole embryos up to stage NF40 (early larval stages), each deiodinase gene showed a distinct profile of expression. We found maternally expressed D1 mRNA in the eggs, but no detectable D2 and D3 mRNAs expression was observed at this stage. In a recent paper, D2 and D3 mRNAs were detected in *Xenopus tropicalis* embryos at the two-cell stage, indicating that these mRNAs could be maternally provided in this species. During embryogenesis two major waves of D2 mRNA expression are discerned in *Xenopus laevis* (Morvan Dubois et al., 2006): the first around stages NF12 and NF14 (neurula) and the second at stages NF31–NF37 (late embryogenesis). D3 mRNA levels in embryos are fairly constant throughout early embryogenesis, only peaking at stage NF37, whereas D3 mRNA show a three-fold increase. Interestingly, similar results were obtained for D2 and D3 mRNA expression in *Xenopus tropicalis*, while D1 mRNA expression was not investigated in this species (Tindall et al., 2007).

### **1.2. Deiodinases exhibits distinct patterns of expression in early development**

The localisation of deiodinase mRNAs expression could provide clues to their possible role in

organogenesis. *In situ* hybridization reveals strong signals for each deiodinase mRNA from stage 24 onwards in the axial structures of *X. laevis* embryos. However, the different deiodinase gene expression patterns do not fully overlap. Whole mount staining of *X. laevis* embryos (Morvan Dubois et al., 2006, Fig. 2) showed that D1 gene expression was strong in the notochord and the branchial arches at stage NF30 and around the eye and in the brain at stage NF 35. The D2 and D3 mRNA share similar, but not identical, patterns of expression. Their expression is particularly strong around the branchial arch areas and around the eye, but signals are also seen in the spinal chord at stage NF 30. In contrast to D1 expression, D2 and D3 mRNAs are not detectable in the notochord. Interestingly, similar patterns of expression were found for *X. tropicalis* D2 and D3 mRNA, with the exception that they are both expressed in the notochord, but not in the spinal chord (Tindall et al., 2007). The deiodinases expression patterns suggest that TH could play a role in the formation of axial structures and in the maturation of the different sensory organs such as the otic vesicle and the eye. This latter result corroborates the demonstration that unliganded TR are essential to eye development (see below, Havis et al., 2006), and this role could be highly stage- and cell-type specific.

A striking observation is that in both species, the expression patterns show rapidly evolving changes during embryogenesis (Morvan Dubois et al., 2006). This suggests an active role for deiodinases in morphogenic dynamics, especially in axial and cephalic structures. In the trunk, expression is localized in the axial structures, particularly in the notochord and spinal chord, during neurulation (stages NF20-NF30) and then slightly decreases as the embryo/larva transition is reached (NF37). From late neurula stages all the deiodinase mRNAs are expressed most abundantly in the head region and expression increases up to the embryonic/larva transition. At the embryo/larva transition there is a peak of expression in the cephalic area. Expression of the D1 and D2 deiodinases then decreases slightly at stage NF37 and more markedly at stage NF40 (Morvan Dubois et al., 2006). In contrast, at stage NF35-NF40, there is a peak in D3 expression. The change in the distribution of deiodinases expression suggests a



precise control on the timing of TH local availability.

### **I.3. Deiodinases are active during development**

Deiodinases are selenoenzymes and so require a specific cellular context to be properly translated and processed. D3 functionality was demonstrated both in *Xenopus* embryo and larvae. D3 activity has been shown to be essential to protect a subset of cells during eye development during the larval stages (Marsh-Armstrong et al., 1999). More recently we showed using IOP that D3 is also essential to eye development during the embryonic stages (Havis et al., 2006). It has been suggested that *Xenopus* embryos are not competent to respond to exogenous  $T_3$  (Huang et al., 1999; Cai and Brown, 2004). However, exogenous  $T_3$  inactivation by D3 could well be implicated in the absence of  $T_3$  effects during embryogenesis. Nevertheless, when D3 is inactivated by IOP, there are morphological changes in *Xenopus* embryonic development (Havis et al., 2006), but the combination of  $T_3$  and IOP greatly enhances the developmental defects. These observations, whilst confirming the role of unliganded TR in eye development, challenge the hypothesis that TH could play a general role during early embryogenesis, but do not rule out the possibility of very local, cell-specific responses. Indeed, Oofusa et al. (2001) report that the GFP construct based on the  $TR\beta$  regulatory region is expressed during the neurula stages in axial structures. This result reflects the endogenous expression of  $TR\beta$  mRNA. One hypothesis would be that the expression is correlated with the presence of TH, in a subset of cells during development. This construct could then be interpreted as a TRE-GFP reporter construct displaying  $T_3$ -dependent GFP expression. More recently, we also saw TH responses in early transgenic embryos (Fini and Demeneix, in preparation).

The functionality of activating deiodinases was assessed in stage NF35 embryos, by comparing deiodination assays from extracts of head and trunk regions, as the D1 and D2 mRNAs are expressed at higher levels in the head than in the trunk. We thus tested the hypothesis that head structures of stage NF35 *Xenopus* embryos express 5' deiodination activity. The assays showed

that stage NF35 embryo heads contain significantly higher  $T_4$  ORD activity than the trunk region. At this stage, the embryonic *Xenopus* tissues had predominantly ORD activity, as indicated by the roughly equivalent generation of radioactive  $T_3$  and iodide from  $[3',5'-^{125}I]$   $T_4$ . Most of the  $T_4$  ORD activity observed in the embryonic *Xenopus* tissues represent predominantly D2 activity, since it was affected by specific inhibitors and saturated at much lower  $T_4$  concentrations than those required to saturate the recombinant xID1 (Morvan Dubois et al., 2006).

#### **1.4. $T_3$ and $T_4$ are present in the egg and early embryo**

Given that functional deiodinases are found in embryos and early larval stages, prior to thyroid organogenesis, we used radio-immunoassay to investigate if their substrates,  $T_3$  and  $T_4$ , were found in *X. laevis* eggs and embryos. This question needed to be addressed, as TH cannot be provided by food before stage 45 when the tadpoles begin to feed. First, we found that neither  $T_4$  nor  $T_3$  were found in the egg matrix envelope. Second, we observed a large variability of TH content in eggs laid by different females (Morvan Dubois et al., 2006). To address the question of the metabolism of TH during embryogenesis we compared levels in eggs and NF35-37 embryos from the same females. Measurable, physiological relevant amounts (ie, in the order of magnitude compatible with the  $K_m$  of deiodinases) in the femtomole range, of both hormones were found in eggs and embryos. There were no significant variations in  $T_4$  levels between eggs and embryos (Fig. 3A). In contrast, a significant difference ( $p < 0.05$ ) was observed between the mean of  $T_3$  measured in eggs and that found in NF 35-37 embryos.  $T_3$  is increased (three fold) at stage 35-37 compared to eggs (Fig. 3B; (Morvan Dubois et al., 2006)), suggesting a conversion of  $T_4$  into  $T_3$  by the embryonic activating deiodinases.  $T_3$  and  $T_4$  have been reported in the vitellus of different amphibian species in previous studies (Volpert et al., 1958; Gancedo et al., 1997). Further, it is well documented that TH also accumulates in the avian yolk (Roche et al., 1957), and  $T_3$  response signals were found in early transgenic chick embryos (Flamant and Samarut, 1998). An interpretation would be that  $T_4$  in the avian and *Xenopus* embryos is exclusively of maternal origin. However, Tindall et al (2007) have shown that thyroid peroxidase

(TPO) is also expressed during embryonic development. However, up to now no functional study exist to show whether this enzyme is active during *Xenopus* development. Nevertheless, expression patterns of TPO during *Xenopus* embryogenesis are similar to those observed for deiodinases. Taken together, these findings bolster the hypothesis that TH could play a role in *Xenopus* embryogenesis.

## **II. Dual role of TR during development: tissue specific repression followed by activation**

### **II.1. The dual role hypothesis**

The amino acid sequences of the TRs are highly conserved through evolution. In mammals there are two types of TR ( $\alpha$  and  $\beta$ ), whereas in *Xenopus laevis*, there are two TR $\alpha$  (A and B) and two TR $\beta$  (A and B) due to pseudo-tetraploidy (Yaoita et al., 1990). In *Xenopus laevis*, TR $\alpha$  and TR $\beta$  are differentially regulated during development. Fig. 4A shows a qualitative representation of T<sub>3</sub> concentration and TR mRNA contents during amphibian development. Their mRNAs are present at very low levels in the oocyte and during embryogenesis (Banker et al., 1991; Oofusa et al., 2001; Havis et al., 2006). High levels of TR $\alpha$  mRNA are present after hatching (stage NF35/38) and until the end of metamorphosis when levels decrease markedly, staying low in juveniles and adults (Yaoita and Brown, 1990). TR $\beta$  mRNA levels increase in parallel with endogenous TH levels (Yaoita and Brown, 1990). TR $\beta$  genes were found to be direct response genes (Ranjan et al., 1994; Machuca et al., 1995). As for TR $\alpha$  mRNA, TR $\beta$  mRNA levels decrease in juveniles and adults (Yaoita and Brown, 1990). Similar profiles were observed when analysing protein expression (Eliceiri and Brown, 1994). Furthermore, the proteins are functionally active as shown by T<sub>3</sub> treatment inducing precocious metamorphosis (Yaoita and Brown, 1990).

The *Xenopus laevis* TRs behave similarly to the mammalian and avian TRs in terms of their secondary structure organization, ligand and DNA binding properties, and their mechanism of

action (Wong and Shi, 1995). In 1997, a model was proposed to explain the mechanism of repression and activation of transcription by TR (Fig. 4B, adapted from Wolffe et al 1997). In absence of  $T_3$ , *Xenopus* TR and RXR (Retinoid X receptor) bind  $T_3$ RE and recruit a corepressor complex that contains histone deacetylase activity (Sachs et al., 2002). Histone deacetylation leads to a closed chromatin conformation inaccessible for transcriptional machinery. Other effects involving chromatin remodelling or direct interaction with transcriptional machinery could also be involved.  $T_3$  binding induces a conformational change of TR that relieves its inhibitory effect with first, the release of the corepressor complex and second, the recruitment of a SRC/p300 coactivator complex that contains histone acetyl transferase activity. These histone modifications lead to chromatin opening. TRs can also recruit coactivator complexes such as the SWI/SNF complex involved in chromatin remodelling and the Mediator complex directly involved in transcription activation (Huang et al., 2003).

The expression profiles, together with the ability of TR to both repress and activate TH-inducible genes in the absence and presence of TH, respectively, suggest dual functions for TRs during development (Fig. 4). That is, before metamorphosis, TRs act to repress  $T_3$ -response genes. As many of these genes are likely to participate in metamorphosis their repression by unliganded TR will help to prevent premature metamorphosis and ensure a proper period of tadpole growth. As the thyroid gland matures,  $T_3$  is synthesized and secreted into the plasma transforming TRs from repressors to activators, and inducing the expression of  $T_3$ -response genes, including the  $TR\beta$  genes, thus leading to metamorphosis. The expression profile of some  $T_3$ -response genes is in agreement with the dual role hypothesis. These genes such as Sonic hedgehog (xhh) and stromelysin 3 (ST3) are first activated during *Xenopus laevis* organogenesis. Their mRNA levels are subsequently down regulated at the beginning of larval stages coinciding with the increase of  $TR\alpha$  mRNA levels and up regulated again during metamorphosis when  $T_3$  is synthesized (Stolow and Shi, 1995; Patterson et al., 1995). Moreover, TR and RXR over-expression during embryogenesis regulates efficiently and specifically ST3 and xhh, repressing them in the

absence of  $T_3$  but activating them when  $T_3$  is present (Puzianowska-Kuznicka et al., 1997). However, the physiological relevance of this dual role is unknown. Thus, considering the importance of TR function in *Xenopus laevis* development, this model provides a good system to address this question.

## II.2. Repression by unliganded TR during larval growth

Analyses of metamorphic  $T_3$ -target genes indicate that the majority are not expressed during larval development prior to metamorphosis. Nevertheless, some of them, including TR $\beta$ , are expressed at low levels in early development. The hypothesis of a dual role for TR during amphibian development suggests that  $T_3$ -response gene repression is active and TR dependent. If this is the case, we would expect TR to be expressed and found at target  $T_3$ REs during larval growth. ChIP studies have shown that TR and RXR do bind  $T_3$ -response gene promoters, during larval development, independently of the presence of  $T_3$  (Fig. 5A; (Sachs et al., 2000; Havis et al., 2003)).

Further, several lines of evidence suggest that corepressors are recruited at the promoters of  $T_3$ -response genes in absence of  $T_3$ . First, over-expression of a dominant negative NCoR led to increased transcription from a  $T_3$ -dependent promoter (Fig. 5B; (Sachs et al., 2002)). Second, association of NCoR with the promoter of  $T_3$ -response genes was shown by ChIP assay in premetamorphic tadpole tail and intestine, using an antibody against NCoR (Sachs et al., 2002). In the absence of  $T_3$  but not in its presence,  $T_3$ RE containing regions of  $T_3$ -response genes were highly represented in the DNA fragments immunoprecipitated by NCoR antibody (Fig. 5C). Thus, NCoR recruitment at  $T_3$ -response genes in the absence of ligand is in accordance with the classical TR action model (Fig. 4B). Third, ChIP assays with acetylated histone H4 antibody showed that H4 histone acetylation at  $T_3$ -response gene promoters was increased by  $T_3$  treatment of premetamorphic tadpoles (Sachs et al., 2000; Havis et al., 2003). This result suggests that in premetamorphic tadpoles, where TH levels are low, histones H4 are deacetylated. Moreover, following  $T_3$  treatment, the increase of H4 acetylation suggests that

HDAC activity is no longer effective at the promoter, probably due to its release with NCoR. To further analyze the role of HDAC in transcriptional repression of  $T_3$ -response genes, pre-metamorphic tadpoles were treated with trichostatin A (TSA), an HDAC inhibitor (Yoshida et al., 1990; 1995). TSA treatment of tadpoles increased histone H4 acetylation at  $T_3$ -response gene promoters and was also shown to increase mRNA levels of  $T_3$ -response genes (Sachs et al., 2000; Sachs et al., 2001a; Sachs et al., 2001b).

### II.3. Unliganded TR and embryogenesis

As summarized above, previous studies led to the hypothesis that before the production of  $T_3$  by the endogenous thyroid, the presence of unliganded-receptor is essential for premetamorphic tadpole growth. To test this hypothesis functionally, we generated a *Xenopus laevis* TR $\beta$  mutant (TR188K) construct, that is ineffective for gene repression due to impaired co-repressor NCoR recruitment (Havis et al., 2006). ChIP assay analysis revealed that TR188K binds to T3RE in chromatin *in vivo* (Fig. 6A; (Havis et al., 2006)) and loses its potential to recruit corepressor (Fig.6A; (Havis et al., 2006)). As a consequence, the TR188K form has lost most of its capacity to repress transcription but CHIP and functional assays showed that it is also impaired for transcriptional activation (Fig. 6B; (Havis et al., 2006)). To analyse the requirement of TR mediated repression during development, the effects of GFP, TR, TR188K and TR $\Delta$ AF2 over-expression on embryos and tadpoles before  $T_3$  production occurs was compared using germinal transgenesis. TR $\Delta$ AF2, that is only impaired for transcriptional activation, was used to distinguish whether TR188K effects were the result of repression or activation defects. Over-expression of TR188K was found to be lethal during embryonic development, killing 75% of embryos. TR188K over-expression in surviving embryos affected the normal morphological development (Havis et al., 2006). To refine the analysis, we focused on the head region where TR $\alpha$  is ubiquitously expressed and TR $\beta$  is expressed in the neural tube and the retina (Fig.6C; (Havis et al., 2006)). Most GFP (control), TR and TR $\Delta$ AF2 transgenic embryos displayed normal

head morphology with correctly formed eyes, showing no implication of the TR activating function in the TRI188K phenotype. However, TRI188K embryos had abnormal head morphologies either lacking, or with underdeveloped, eyes (Havis et al., 2006). To confirm the lack of eye structure in TRI188K transgenic embryos whole mount *in situ* hybridization analysis was carried out with probes against *Pax6*, *Rx*, *Otx2* and *xhh* mRNA, four genes involved in eye development (Harris and Perron, 1998). Whole mount ISH showed these genes to be expressed in or around the developing eye of GFP, TR and TR $\Delta$ AF2 transgenic embryos, but there were no or only weak signals in the heads of TRI188K embryos (Fig. 6D; (Havis et al., 2006)).

The physiological function of unliganded TR could be questioned because physiological T<sub>3</sub> treatment of embryos has no effect on early embryogenesis (Puzianowska-Kuznicka et al., 1997). One explanation could be the presence of the inactivating deiodinase D3 (Marsh-Armstrong et al., 1999), 1999; (Morvan Dubois et al., 2006) degrading any T<sub>3</sub> present or introduced. Indeed, in the retina, *D3* mRNA starts early, being concomitant with TR $\beta$  expression. Functional deiodinase activity can be revealed by using the deiodinase inhibitor iopanoic acid (IOP). IOP treatment results in eye malformation (Morvan Dubois et al., 2006). Interestingly, whole mount *in situ* hybridization with *Pax-6*, *Rx*, *Otx2* and *xhh* probe revealed that IOP treatment decreases their expression in or around the eye, similar to that observed with over expression of TRI188K (Fig. 6E; (Havis et al., 2006)). As IOP treatment inhibits both activating and inactivating deiodinases, embryos were treated with both T<sub>3</sub> and IOP. A phenotype due to IOP will be rescued by T<sub>3</sub> treatment if T<sub>3</sub> production is implicated. In contrast, T<sub>3</sub> treatment will aggravate a phenotype where IOP affects mainly T<sub>3</sub> degradation. Combining IOP and T<sub>3</sub> led to a stronger effect on eye shape (Havis et al., 2006). These results thus bolstered first our hypothesis that unliganded TR are essential for normal eye development, and second, that T<sub>3</sub> inactivation by deiodinase in the eyes could explain the lack of effect of adding exogenous T<sub>3</sub> during embryogenesis.

To target more specifically endogenous TR signalling, NH-3, a synthetic T<sub>3</sub> agonist/antagonist

was used to treat embryos. NH-3 binds TR, blocks TR co-activator recruitment but also induces release of co-repressor (Webb et al., 2002). Thus, TR with NH-3 will mimic the TR mutant I188K (a TR that is unable to either repress or activate transcription). Treatment with NH-3 strongly altered embryonic development compared to control embryos with smaller and abnormally formed and positioned eyes (Havis et al., 2006). Finally, molecular analysis by whole mount *in situ* hybridization showed that *Pax6*, *Rx*, *Otx2* and *xhh* expression is weaker and the localisation different in NH-3 treated embryos compared to untreated embryos (Fig. 6E; (Havis et al., 2006)). Our data thus provide *in vivo* evidence that *TR $\beta$*  is expressed and functions in a tissue specific manner during embryogenesis, at stages when the thyroid gland is inactive. The results suggest a new physiological role of unliganded TR, prior to its participation in the repression of metamorphic T<sub>3</sub> response genes during larval development that of eye formation.

## Conclusion:

TRs are present in the *Xenopus* embryos, displaying tissue specific distributions. Similarly all three deiodinases and their substrates are also present in early embryos, bolstering the idea that TH signalling is involved in early embryogenesis. TH is thus implicated in all the major stages and transitions in the frog life cycle (embryo/larvae & tadpole/adult ie hatching and metamorphosis). Our current hypothesis, derived from our recent work and much previously published data from other laboratories would be that TR and TH responses contribute to early vertebrate development implicating cell specific and tissue specific responses. These responses require either activation or repression of TR regulated genes. For instance, TR as an aporeceptor is fundamental for some steps of the developmental program, such as discrete phases of the eye development. Taken together, the dynamic expression patterns of each deiodinase during embryogenesis, the presence of correctly translated/processed deiodinases, the amount of maternally provided TH and the *in vivo* conversion of T<sub>4</sub> to T<sub>3</sub> at early embryonic



stages, suggest that TH signalling contributes to early amphibian development. This is contrary to the previously held hypothesis that TH signalling in amphibians only becomes biologically relevant from premetamorphic stages onwards.

## Legends to Tables and Figures.

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## FIGURE LEGENDS

**Figure. 1: Thyroid hormone activating and inactivating pathway**

Deiodinases are seleno-proteins that catalyze the deiodination of TH. Three deiodinases have been characterized in different vertebrates. Type 2 deiodinase (D2) converts the prohormone  $T_4$  into the receptor-active form  $T_3$  via the outer ring or 5' deiodination (ORD). Type 3 deiodinase (D3) converts, via inner ring or 3 deiodination (IRD),  $T_4$  and  $T_3$  into the inactive forms, reverse  $T_3$  ( $rT_3$ ) and 3,3'-diiodothyronine, respectively. The D1 carries out both ORD and IRD reactions and thus can be seen as either an activating or inactivating deiodinase.

**Figure. 2: Dynamic pattern of D1 mRNA expression during embryogenesis**

mRNAs encoding D1 are expressed in the head region and axis of stage NF26 and NF35 *X. laevis* embryos. *In situ* hybridization on whole mount embryos was performed with specific probes for D1, A,B : stage NF 26, C: stage NF 35. The expression pattern is very dynamic during embryonic development, as the D1 signal has a marked expression in notochord at stage NF26 but at this signal is stronger in neurogenic areas and dorsal fin at stages NF35. ba: branchial arches, df: dorsal fin Nt : notochord, v : ventricular zone. Bar = 50 mM .

### Figure 3. T<sub>3</sub> levels increase during *Xenopus* embryogenesis

RIA measurements of T<sub>3</sub> and T<sub>4</sub> in extracts from five different batches of eggs and five corresponding batches of embryos. The maximum and minimum values are represented by the bars, and the 95% confidence interval is boxed. The median value is represented by an horizontal bar in the box. The T<sub>4</sub> content is not significantly different between the eggs and the embryos, and there is large variability in each group. In contrast, T<sub>3</sub> content in eggs is low with little variability, and it significantly increases in embryos ( $p=0,024$ ). Total amounts of TH were normalized by the number of eggs/embryos in each sample. The weighted means of the different measures for the same batch of eggs (ie same female, same condition) was considered as one sample. Differences were analysed by one-tailed paired t test ( $n=5$ ,  $r=0.88$ ).  $*=p<0.05$ .

### Figure. 4: Dual functions of thyroid hormone receptor during amphibian development.

A) Developmental levels of TH and expression of TR $\alpha$  and TR $\beta$  throughout embryogenesis, tadpole growth and metamorphosis. Stages of development are indicated according to the table of *Xenopus laevis* development (Nieuwkoop and Faber, 1956). In the *Xenopus* tadpole, thyroid gland organogenesis begins around stage NF40 and iodine uptake is detectable by stage NF46, coincident with histological signs of secretory activity. Plasma T<sub>3</sub> concentrations are from Leloup and Buscaglia (1977). mRNA levels for TR $\alpha$  and TR $\beta$  are based on data from Yaoita and Brown (1990). For clarity, T<sub>3</sub> and mRNA levels are plotted as arbitrary units on different scales. For

each major step of development, the state of  $T_3$ -response gene transcription is indicated in correlation with current knowledge on TR mechanism of action (repression versus activation). Measurable levels of  $T_4$  and  $T_3$  are found in the plasma of stage NF52 animals but not from earlier larval stages. From this and the apparent insensitivity to  $T_3$  treatment of the embryos before stage 45, it was generally assumed that TH signalling during amphibian development becomes relevant only in pre-metamorphic stages (NF 45 to NF 57). B) A proposed mechanism for transcriptional regulation by TR. TR is a transcription factor that binds as heterodimer with RXR (retinoid X receptor) to the  $T_3$ RE ( $T_3$  response element) in target gene. In absence of  $T_3$ , unliganded TR represses gene transcription likely through the recruitment of corepressor complex containing NCoR and histone deacetylase. The chromatin is then closed and the transcription is repressed. Upon  $T_3$  binding, a conformational change takes place in the heterodimer. Liganded TR releases the corepressor complex and can recruit several coactivator complexes. They function in part through chromatin modification and remodelling, opening the chromatin for full activation of target genes.

**Figure. 5: Unliganded TR and larval development.**

Unliganded TR binds DNA and recruits corepressors to repress  $T_3$ -response gene expression during larval development. A) Stage-dependent effects of  $T_3$  on transcription, high DNA binding by TRs at  $T_3$  response genes. First, premetamorphic tadpoles but not embryos are competent to respond to  $T_3$  treatment.  $TR\alpha$  mRNA expression increases between embryonic and tadpole stages.  $TR\beta$  was also used as  $T_3$ -response gene. Stage 20 embryos and stage 47 tadpoles were treated with 100 nM  $T_3$  for 24 h. Total RNA was extracted from whole animals and used for PCR analysis of TR gene. PCR products were resolved on agarose gels, and the gels were stained with ethidium bromide. The expression of the ribosomal protein L8 (rpl8) was used as an internal control because its mRNA levels are not affected by  $T_3$  (Shi and Liang, 1994). Second, increased levels of TR expression between embryonic and tadpole stages correlate with

increased TR binding to TR target genes. Moreover, TR binding to T<sub>3</sub>REs was not affected by T<sub>3</sub>. Chromatin from stage 20 embryo and stage 47 tadpole nuclei was immunoprecipitated with antibodies against TR and analyzed by PCR for the presence of the fragments containing the T<sub>3</sub>RE of TR $\beta$  gene. Aliquots of the chromatin before immunoprecipitation were used directly for PCR as control (input). B) Requirements of corepressor for TR-mediated gene repression during larval period. Dominant negative NCoR (CoRNR) abolishes the repression of T<sub>3</sub>RE-containing promoter by unliganded TR. Over expression of CoRNR, but not the control peptide (Ct) increases the expression level of firefly luciferase expression vector driven by a T<sub>3</sub>RE. Over expression was realized by *in vivo* gene transfer (de Luze et al., 1993). *Xenopus laevis* dorsal muscles were co-injected with 0.5  $\mu$ g of vector expressing CoRNR or control vector, 0.5  $\mu$ g of T<sub>3</sub>RE-tk-firefly luciferase vector and 0.1  $\mu$ g of SV40-Renilla luciferase vector as an internal control for transfection efficiency. Two days later, the luciferase activities were assayed from muscle homogenates. The ratio of the firefly luciferase over *Renilla* luciferase was plotted as fold induction with standard errors. Each point represents at least seven tadpoles. Statistical significance was tested using Student's t test. \*\* Significant change with  $p < 0.001$ . C) Chromatin Immunoprecipitation (ChIP) assay show that NCoR is released from T<sub>3</sub>-response gene promoter following T<sub>3</sub> treatment. Pre-metamorphic tadpole tail nuclei were isolated after 10 nM T<sub>3</sub> treatment for 48 h. Chromatin was immunoprecipitated with anti-NCoR antibody. Finally, the precipitated DNA is analyzed by PCR as described for the presence of T<sub>3</sub>-response gene promoters such as TR $\beta$  promoter. EF1 $\alpha$ , which is not regulated by T<sub>3</sub>, serve as a negative control.

**Figure. 6: Unliganded TR and embryonic development.**

A) TRI188K mutant is impaired for both transcriptional repression and activation. First, over-expression, in tadpole brain, of TR, but not TRI188K decreases the expression of T<sub>3</sub>RE-tk-Luc. TR, TRI188K and empty vectors (all 0.5  $\mu$ g) were coinjected with 0.5  $\mu$ g of the T<sub>3</sub>RE-tk-Luc. The

plasmids were complexed with polyethylenimine. Second,  $T_3$  does not affect expression of T3RE-tk-Luc in presence of TRI188K. One  $\mu$ g of TRI188K vector was coinjected with 0.2  $\mu$ g of T3RE-tk-Luc. Following injection, tadpoles either received 10 nM  $T_3$  (+ $T_3$ ) for 48h, or were used as controls (- $T_3$ ). For all the experiments, luciferase was assayed 48h post-transfection, and expressed as % of the Ct value (100%) or fold induction with - $T_3$  value equal 1. Means  $\pm$  S.E.M. are given. Each point represents at least seven animals. Significant changes (\*\*:  $p < 0.01$ ) are indicated as compared to Ct or - $T_3$  value. B) The TRI188K mutant binds DNA and is impaired for NCoR corepressor recruitment. Two day-old transgenic embryos nuclei were isolated and subjected to ChIP assays with antibodies against GFP or NCoR. All the exogenous TR forms are fused to GFP to distinguish them from endogenous TR. ChIP products were analysed by PCR for the presence of T3RE-containing fragments of *TR $\beta$*  promoter. Aliquots of chromatin before immunoprecipitation were used for PCR as controls of DNA quantity (Input). Each transgenic group represented 100 embryos. C) *TR $\beta$*  gene transcripts are present during embryonic development. *TR $\beta$*  expression was examined at stage NF32 by whole mount ISH. Red arrows and black arrows indicate respectively expression in the eyes and neural tube. D) Effects of TRI188K over-expression on *Pax6*, *Rx*, *Otx2*, and *xhh* gene expression in transgenic embryos. Gene expression followed by whole mount ISH in the head for one stage NF28 embryo representative of the major phenotype observed for each type of transgene used. E) Effects of IOP and NH<sub>3</sub> on embryonic development. *Pax6*, *Rx*, *Otx2*, and *xhh* expression at stage NF28 revealed by whole mount ISH in the head for one embryo representative of the major phenotype following IOP or NH<sub>3</sub> treatment. Embryos at stage NF11-12 were treated for 24h with 5  $\mu$ M IOP in 0.1% ethanol. Embryos at morula stage were treated (48h) with either 0.1% ethanol, 10  $\mu$ M NH<sub>3</sub>, or were treated with 0.1% ethanol (Ct).

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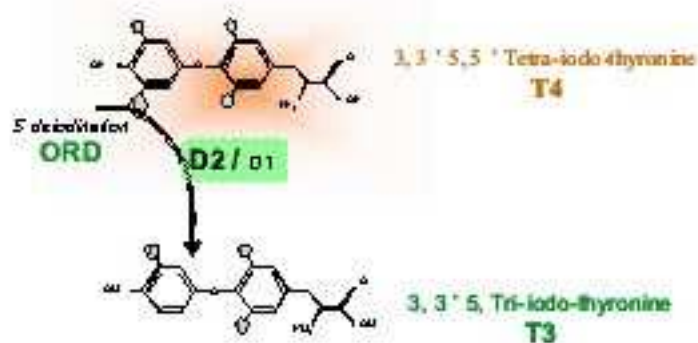
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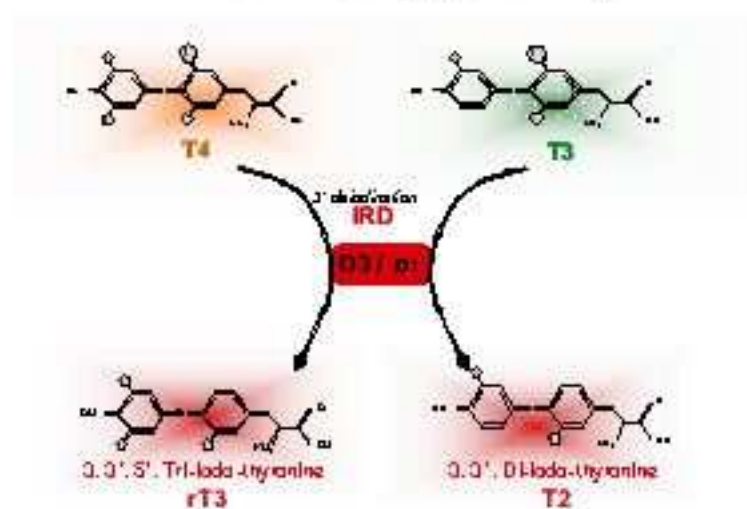
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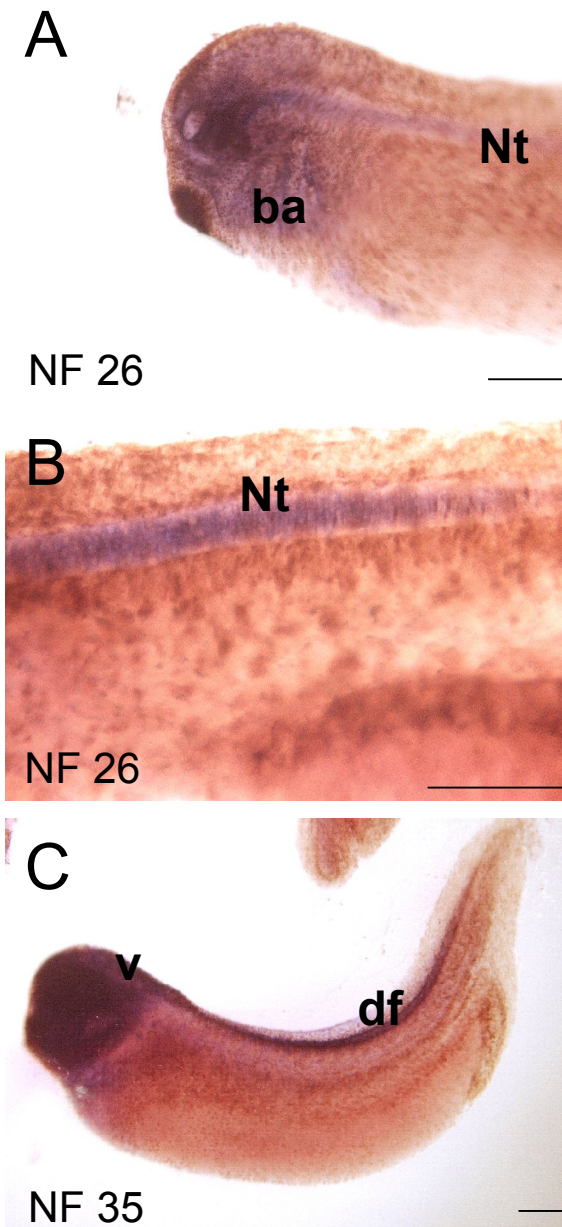
## Activating pathway



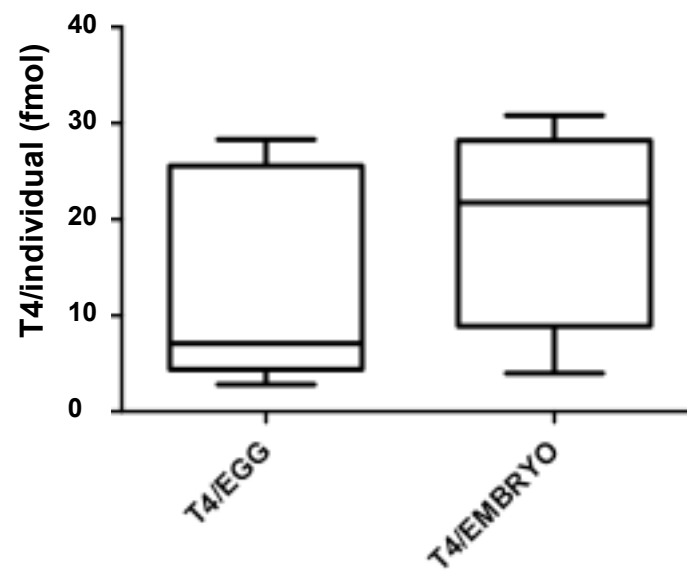
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## Inactivating pathway





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