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Review

Mechanisms of thyroid hormone receptor action during development: Lessons from amphibian studies☆

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ABSTRACT

Background: Thyroid hormone (TH) receptor (TR) plays critical roles in vertebrate development. However, the in vivo mechanism of TR action remains poorly explored.
Scope of review: Frog metamorphosis is controlled by TH and mimics the postembryonic period in mammals when high levels of TH are also required. We review here some of the findings on the developmental functions of TH and TR and the associated mechanisms obtained from this model system.
Major conclusion: A dual function model for TR in Anuran development was proposed over a decade ago. That is, unliganded TR recruits corepressors to TH response genes in premetamorphic tadpoles to repress these genes and prevent premature metamorphic changes. Subsequently, when TH becomes available, liganded TR recruits coactivators to activate these same genes, leading to metamorphic changes. Over the years, molecular and genetic approaches have provided strong support for this model. Specifically, it has been shown that unliganded TR recruits histone deacetylase containing corepressor complexes during larval stages to control metamorphic timing, while liganded TR recruits multiple histone modifying and chromatin remodeling coactivator complexes during metamorphosis. These complexes can alter chromatin structure via nucleosome position alterations or eviction and histone modifications to contribute to the recruitment of transcriptional machinery and gene activation.
General significance: The molecular mechanisms of TR action in vivo as revealed from studies on amphibian metamorphosis are very likely applicable to mammalian development as well. These findings provide a new perspective for understanding the diverse effects of TH in normal physiology and diseases caused by TH dysfunction. This article is part of a Special Issue entitled Thyroid hormone signalling.

1. Introduction

Thyroid hormones (TH) regulate multiple developmental and physiological functions in vertebrates. At the cellular level, 3,5,3′ triiodothyronine (T_3), the active form of TH, controls cell metabolism, proliferation, and commitment to differentiation or apoptosis. TH is synthesized in the thyroid gland. This organ has attracted the attention of physicians since antiquity. It was only 100 years ago that TH had begun to be characterized. In 1912, Friedrich Gudernatsch fed premetamorphic tadpoles with several horse organs and found that thyroid gland but none of the others could accelerate amphibian metamorphosis [1].

Amphibian development is a biphasic process. Embryonic stages and juvenile life are separated by a larval (tadpole) period that ends with metamorphosis. Metamorphosis is a switch that results in the reprogramming of the morphological and biochemical characteristics of nearly all tadpole organs, including de novo development (limbs), tissue remodeling (nervous system) and organ resorption (tail) [2]. These transformations involve apoptosis of larval cells and concurrent proliferation and differentiation of adult cell types.

Amphibian metamorphosis bears strong similarities with the perinatal (postembryonic) development in mammals at molecular and morphological levels [3]. First, both take place in a period when plasma TH levels peak during development. Second, both mammals and

Abbreviations: TH, Thyroid hormone; TR, Thyroid hormone receptor; T_3, 3,5,3′ triiodothyronine; TRE, TH response element; DR, direct repeat; IR, inverted repeat or palindrome; ER, everted repeat; RXR, retinoic acid X receptor; dp, dominant positive; dn, dominant negative; NCoR, Nuclear receptor CoRepressor; SMRT, Silencing Mediator for RAR and TR; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; TBL1, transducin beta like protein 1; SRC, steroid receptor coactivator; CBP, p300/CREB binding protein; CARM1, coactivator associated arginine methyltransferase 1; PRMT1, Protein arginine methyltransferase 1; DRIP, vitamin D receptor interacting protein complex; TRAP, TR associated protein complex; ARC, activator recruited cofactor complex; BRG1, Brahma related gene 1; BAF57, BRG1 associated factor 57; HDM, histone demethylase; HAT, histone acetyltransferase; HMT, histone methyltransferase; ezh2, enhancer of zeste 2; H3, histone H3; H4, histone H4; Me2, Dimethyl; Me3, Trimethyl; K, Lysine; R, Arginine; X. laevis, Xenopus leavis; X. tropicalis, Xenopus tropicalis

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(most) anurans change their living habitat during this period, from an aquatic (amniotic) to a terrestrial environment. Third, many processes that occur during anuran metamorphosis resemble those occurring during postembryonic development in mammals [4,2]. These include skin keratinization, the induction of urea cycle enzymes, the switching of the hemoglobin genes from larval (tadpole) or fetal types to frog or adult types, respectively, and the developmental progression and restructuring of the central and peripheral nervous systems, etc. One of the best studied among them is the development of the adult intestine. Recent studies have shown that both the metamorphic intestinal remodeling in *Xenopus laevis* (X. laevis) and postembryonic intestinal maturation in mouse are dependent on TH and involve the formation of adult stem cells from pre-existing tadpole/neonatal intestinal epithelial cells [5,6]. Such findings suggest conserved molecular mechanisms governing a TH-dependent postembryonic development in vertebrates. Thus, amphibian metamorphosis can serve as a model to investigate the effects of TH on vertebrate development. More importantly, although TH is clearly important for embryogenesis in humans and other mammals, it has been difficult to investigate the mechanisms in mammals. This is in part due to the difficulty to manipulate the uterus-enclosed mammalian embryos and to separate the direct effects of TH on embryos from the indirect maternal effects caused by TH. The metamorphic changes in tadpoles occur in a free-living organism and can be easily controlled by adding TH or its inhibitors to the rearing water.

The diverse effects of TH suggest the existence of tissue specific and developmental stage specific control of gene expression by TH to coordinate different transformations in various organs. TH can affect gene expression by binding to TH receptors (TRs). TRs are transcription factors that belong to the superfamily of nuclear receptors [7,8]. TH is a versatile player, not only upregulating the expression of some genes but also downregulating expression of other genes. It is generally believed that both effects are mediated by TR. Studies on gene repression by TR in the presence of TH are very limited [9]. To date, most of the studies on the mechanisms of TR action have been carried out on positively regulated TH response genes. Extensive in vitro and cell culture analyses have shown that TRs bind to specific sequences present in the promoter regions of their target genes to regulate their expression. Molecular and genetic studies on postembryonic development in frogs coupled with in vivo analysis in the reconstituted frog oocyte transcription system have provided important insights on transcriptional activation and repression by TR during vertebrate development.

2. Gene regulation by thyroid hormone receptor

During the sixties, J.R. Tata showed that TH induced mRNA and protein synthesis in mammals [10] as well as in amphibians [11]. Several studies in mammals subsequently showed the existence of nuclear binding sites for TH [12] that led to the concept of TR. Later, the isolation of complementary DNA (cDNA) coding for *verbA* oncogene from avian erythroblastosis virus led to the cloning of the proto-oncogene *cerbA* that was identified as a TR [13,14]. A few years later the *X. laevis* TR, first from an amphibian, was cloned [15,16].

2.1. Amphibian thyroid hormone receptors

As in mammals there are two types of TR, α and β [16–20]. The amino acid sequences of the amphibian TRs are well conserved in evolution compared to their homologs described in mammals, fishes and chickens. Additionally, alternative splicing yields multiple mRNA isoforms similar to those found in mammals [16].

Xenopus TR, as most other nuclear receptors, can be organized from the amino to the carboxyl terminus into four domains that indicate differing functions, A/B, C, D and E; however, there can be overlap between the functions of each domain. The A/B domain is generally involved in the transcriptional activation and is highly variable in sequence and length, the shortest being in *X. laevis* TRβ1 where this domain is absent [16]. The recent isolation of TRβ2 from *Xenopus tropicalis* (X. tropicalis) led to the identification of an A/B domain with high sequence identity to the mammalian domain [LM Sachs unpublished data], further confirming the conservation of this domain's function in some amphibian isoforms.

The C domain is one of the signatures of all nuclear receptors. This highly conserved region is involved in DNA binding and thus in the specific recognition of TH response elements (TRE) present in TH response genes. The consensus TRE consists of two repeats of the hexameric AGGTCA sequence. In mammals, this half site can be configured in direct repeat (DR), inverted repeat or palindrome (IR) and everted repeat (ER). The hormonal specificity is dictated by the number of nucleotide spacing the two half sites as well as its direction. A DR with 4 nucleotide spacing most strongly binds to TR and is thus considered the highest affinity TRE. However, an IR with no nucleotide between the two half sites or an ER with 6 or 8 nucleotide spacing can also function as a TRE. In Xenopus, all the identified TREs are DR4 [21]. TRs bind to DNA as monomer, homodimer or heterodimer. The most frequent partner is the retinoic acid X receptor (RXR), a nuclear receptor that binds 9-cis retinoic acid. As in mammals, three heterodimeric partners (RXRα, RXRβ and RXRγ) have been cloned in Xenopus [22,23]. Binding of RXR ligand is not necessary for heterodimerization with TR or response to TH. However, RXR is a critical requirement for the developmental function of TR in amphibian. Without RXR, TR alone has little effect on TH response gene transcription in vivo [24].

The D domain is a hinge region that influences surrounding domains C and E and is involved in nuclear localization. The E domain is a complex region with multiple functions. Aside from its important role in ligand binding, it is also involved in the receptor dimerization (interaction with RXR), transcriptional activation in the presence of ligand and transcriptional repression in the absence of ligand. The E domain also interfaces with transcriptional machinery.

Thus, the amphibian TR behaves similarly to the mammalian and avian TR in terms of their secondary structure organization, ligand and DNA binding properties, and their requirement to heterodimerize with RXR for high affinity DNA binding [25]. Likewise, TH action appears conserved in amphibians. TH is versatile and while most direct target genes are upregulated, TH can also suppress gene expression. However, TR can bind to DNA in the presence and the absence of ligand. In the context of a positive TRE, transcription is activated by TH and repressed by TR binding in the absence of TH [26].

2.2. The dual function model

By activating or repressing transcription in a TH dependent manner, TR has dual functions. Considering the importance of TR function in *X. laevis* development, this model has provided a good system to examine the dual function of TR in vivo. Moreover, the relatively rapid and developmentally critical transition from low to high TH levels at the onset of metamorphosis makes Xenopus a unique and important model to study the dual functions of the receptor in vivo. In Xenopus, the duplicated TRα and TRβ genes are differentially regulated during development (Fig. 1; green and dark blue lines, respectively) [29,30]. TRβ genes were found to be direct response genes (Fig. 1) [31,32,20]. Furthermore, TR and RXR genes are coordinately regulated in different tissues during amphibian development [25,20]. The tissue distribution has shown a correlation between TR/RXR expression and organ transformation during metamorphosis [33,30,34]. While TR (such as TRα) is present during both larval and metamorphic periods, T_3 is present only during metamorphosis. Thus, according to the dual function model, TR functions in these two phases are distinct. First, during the larval period, unliganded TR should repress TH response genes (Fig. 1). Second, during the metamorphic period, liganded TR should activate TH target genes

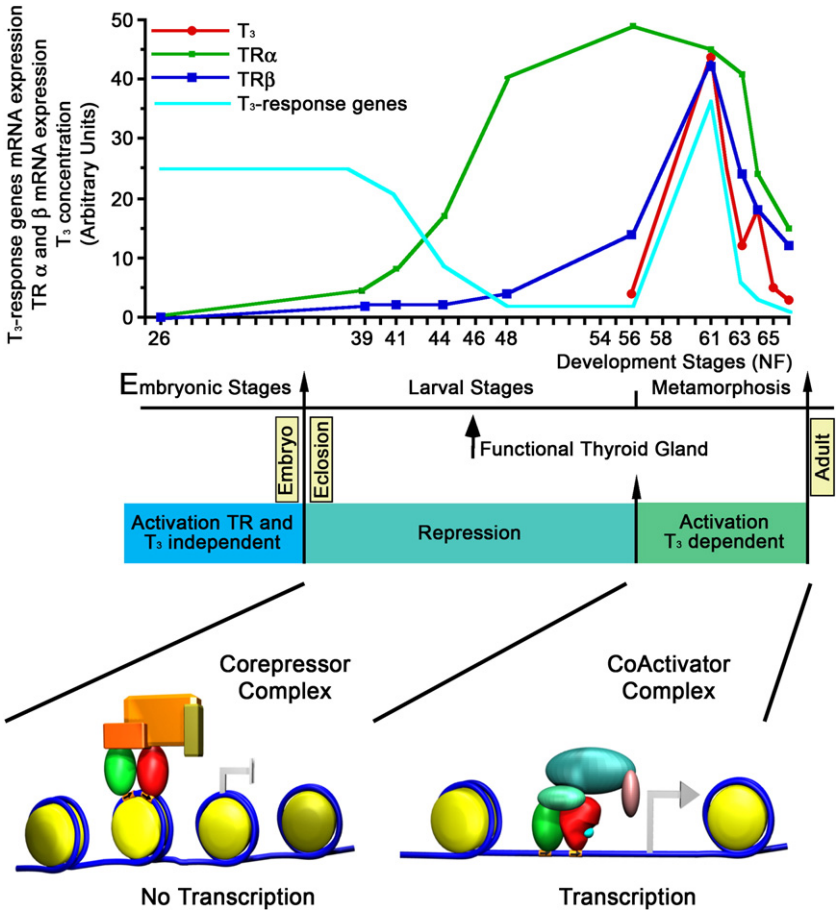

Fig. 1. TR dual function during tadpole development. During embryonic stages of *Xenopus laevis* development, T_3 concentration and the levels of both TR α and β isoforms are low (respectively red, green and blue lines) and those genes characterized as being T_3 responsive at later stages are expressed in a T_3 independent manner (cyan line). After hatching, TRα expression increases progressively, together with the maturation of the thyroid gland, although at these early stages, T_3 concentration remains low. This leads the TR/RXR heterodimers (red/green) bound to TREs (arrows) to recruit the corepressor complex and block T_3 response gene expression, thus preventing premature metamorphosis (lower panel left). During metamorphosis, high T_3 concentrations are associated with strong expression of TRβ. The presence of both TH and TR results in the release of the corepressor complex (orange/yellow) and recruitment of the coactivator complex (light-blue/pink) at TREs (arrows), thus driving T_3 response gene expression (lower panel right). DNA: blue line around, histones octamer: yellow cylinder. Stages of development are indicated according to the table of *Xenopus laevis* development [27]. Plasma T_3 concentrations are from Leloup and Buscaglia [28]. mRNA levels for TRα and TRβ are based on data from Yaoita and Brown [29]. TH response gene mRNA level is indicated in accordance with sonic hedghog expression [45] and stromelysin 3 expression [46].

(Fig. 1) [35,36]. According to this dual function model, during embryogenesis, some TH inducible genes are expressed at a basal level for their role in embryonic organogenesis (Fig. 1; cyan line). In premetamorphic tadpoles, TRs, mostly TRα (Fig. 1; green line), act to repress TH response genes (Fig. 1; cyan line). As many of these genes are likely to participate in metamorphosis, their repression by unliganded TR helps to prevent premature metamorphosis and ensure a proper period of tadpole growth. At the onset of metamorphosis, stimulation of the thyroid gland leads to increased synthesis and secretion of TH into circulation (Fig. 1; red line representing T_3 level). As resultant intracellular TH concentrations increase, T_3 binds and transforms TRs from repressors to activators, which will induce the expression of TH response genes, thus leading to metamorphosis.

Studies in the last two decades have provided several pieces of evidence to support this model. First, many genes have been identified as TH response genes during amphibian metamorphosis [37–44,21]. As previously indicated, some of them are expressed during embryogenesis (Fig. 1; cyan line). Their mRNA levels are subsequently down-regulated at the beginning of larval stages that coincides with the increase of TRα mRNA levels in the absence of TH and up-regulated again during metamorphosis when T_3 is present (Fig. 1; cyan line) [45,46]. Second, TR and RXR are bound to TREs of TH response genes even at larval stages in the absence of T_3 [35,47]. Third, overexpression of TR and RXR in embryos prior to tadpole stages leads to the precocious repression of endogenous TH target genes and the addition of TH can activate these genes in the embryos [24].

More recent molecular and genetic studies provided direct evidence that TR is sufficient for mediating the metamorphic effects of T_3. Transgenic expression of a dominant positive TR (dpTR) that cannot bind to TH but functions as a constitutive activator of TH target genes leads to precocious gene activation and metamorphic transformations just like TH treatment (Fig. 2) [48]. Furthermore, transgenic expression of dominant negative TR (dnTR), which can not bind T_3, blocked TH induced activation of TH target genes and morphological changes (Fig. 2) [49–51]. The correlation between dpTR mediated gene activation and induction of metamorphosis and the dnTR mediated gene repression and inhibition of metamorphosis supports clearly indicates that the metamorphic role of TH is through TR via genomic action of the hormone and not the non genomic action as observed in other cellular and animal contexts [52–54]. Thus, TR is necessary for both preventing premature and at the appropriate time inducing amphibian metamorphosis, supporting the dual function model of TR in development.

3. Coregulator requirement

Direct interactions of TR and RXR with transcriptional machinery were first proposed to explain the mechanism of receptor action.

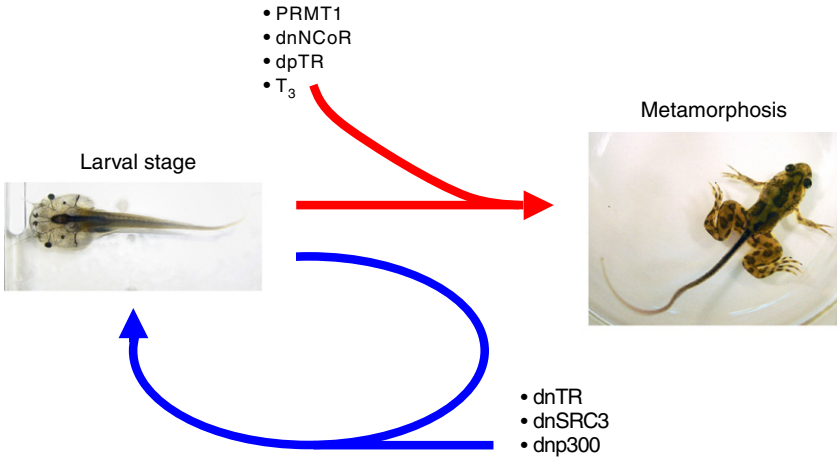

Fig. 2. Various mechanisms that can accelerate or inhibit metamorphosis. Exogenous TH (T_3) can trigger metamorphosis, which can be accelerated in animals overexpressing protein arginine methyltransferase (PRMT1), dominant positive mutants of TRs (dpTR), or dominant negative mutants of NCoR (dnNCoR). This is the result of the derepression and/or activation of TH response genes. Conversely, metamorphosis can be inhibited by overexpression of dominant negative mutants of SRC3 (dnSRC3), p300 (dnp300) or TR (dnTR), leading to the inhibition of TH responsive gene expression.

However, the discovery that proteins could function as corepressors and coactivators implied that instead of signaling to the transcriptional machinery directly, TR might affect gene expression in vivo during development through the recruitment of different cofactors.

3.1. Corepressors

Several corepressor complexes composed of proteins recruited by Xenopus TR in the absence of ligand have been characterized [55–57]. Proteins such as NCoR (Nuclear receptor CoRepressor) and SMRT (Silencing Mediator for RAR and TR) have emerged as important players for unliganded TR mediated gene repression [58]. Both are large proteins (270 kDa) with very similar domain architecture. In the COOH terminal portion, they possess the domain for interaction with TR and other nuclear receptors. This domain contains three I/LXXI/VI motifs (CoRNR boxes) that play a major role in binding with domains D and E of TR [59–62]. In the NH_2 terminal portion, four domains are involved in the transcriptional repression [63–65].

Analyses of TH response gene expression indicate that they are repressed in tadpoles prior to metamorphosis. Our dual function model suggests that this repression is active and TR dependent. Consistently, TR has been shown to bind to TH response gene promoters containing TREs during premetamorphosis as well as metamorphosis [35]. Several lines of evidence have shown that corepressors are recruited at the TREs of TH response genes in the absence of T_3. First, chromatin immunoprecipitation (ChIP) assays have demonstrated the association of NCoR with the TRE of TH response genes in premetamorphic tadpoles [58]. Second, by using in vivo gene transfer in premetamorphic tadpole tail muscle, a dominant negative NCoR was overexpressed. This dominant negative form corresponds to a peptide containing only a CoRNR box of NCoR nuclear receptor binding domain, which could bind to TR to prevent endogenous wild type NCoR from interacting with TR. Overexpression of the peptide led to the loss of repression by unliganded TR and therefore, increased transcription of a TH dependent promoter cotransfected into premetamorphic tadpole tail muscle [58]. Third, transgenic overexpression of a different dominant negative form of TR binding corepressor NCoR (dnNCoR) consisting of its full receptor interacting domain, which could compete away the binding of endogenous NCoR to unliganded TR, led to significant derepression of TH response genes and faster tadpole development than in wild type siblings (Fig. 2) [66].

Several studies have led to the biochemical purification and characterization of NCoR and SMRT complexes associated with histone deacetylase HDAC3, TBL1 (transducin beta like protein 1) and TBL1R (TBL1 related protein) [56,57,67]. Cell culture studies strongly suggest a unique role of NCoR/HDAC3 in repression by unliganded TR in *X. laevis* [68] and in mammals [69]. HDAC3 is a histone deacetylase linking TR mediated gene repression and chromatin structure (see below). In mammals, TBL1 and TBL1R are proteins involved in protein complex recruitment and release with a link to proteasome degradation [70]. Other NCoR complexes have been characterized [55]. One of them is characterized by the presence of HDAC1 instead of HDAC3. In specific contexts (gene or tissue), it is possible that such a HDAC1/NCoR complex could contribute to TR repression as suggested by studies in *X. laevis* [35,47]. First, the association of HDAC1 with the TREs of TH response genes was observed in premetamorphic tadpoles. The release of HDAC1 following T_3 treatment was TH response gene specific [47]. An observation that was also confirmed using a reconstituted transcription system in frog oocyte [68]. For some genes, HDAC1 is released to activate transcription, but for other HDAC1 recruitment is maintained even in the presence of the hormone and high levels of transcription [47]. Recently, this last observation was confirmed at the whole genome level in mammals showing the presence of both histone acetyl transferase and HDAC activity at activated genes [71].

3.2. Coactivators

A larger number of coactivators have been identified to bind TR and transduce transcriptional activation signals in the presence of T_3. Like corepressors, coactivators, such as steroid receptor coactivator (SRC/p160) and p300, form multi-protein complexes [72]. Among them are the three members of the SRC subfamily. One of them, SRC3 was cloned in *X. laevis* and was found to be dramatically upregulated during natural and T_3 induced metamorphosis [73]. SRC3 was recruited in a gene and tissue dependent manner to target genes by TR, both upon T_3 treatment of premetamorphic tadpoles and during natural metamorphosis. At one target gene, SRC3 behaved as described in the current model for TR mechanism of action: recruitment only in the presence of TH [47,74,75]. On another TRE, SRC3 was not recruited in a TH dependent manner in tadpoles, suggesting either no recruitment or constitutive association [47,74]. Interestingly, at the same TRE, SRC3 was recruited only in the presence of T_3 when analyzed in a reporter assay in the frog oocyte [76]. It is possible that cell type specificity or the assay system contributes to this difference. The possible constitutive presence of SRC3 on a TRE but the release of NCoR following T_3 treatment suggests that NCoR is a crucial component of the regulation pathway. This may involve the inhibition of HAT

(histone acetyltransferase) activity, a process that was described in mammals for SMRT complex [77]. Finally, transgenic overexpression of a dominant negative SRC3 [75], whose recruitment in a TH dependent manner blocks the recruitment of endogenous coactivators thereby preventing the TH dependent gene regulation, caused the inhibition of essentially all aspects of TH induced metamorphosis, as well as natural metamorphosis, leading to delayed or arrested metamorphosis (Fig. 2). Interestingly, the release of corepressors was not affected, demonstrating that coactivator recruitment, aside from corepressor release, is required for a TH function in development. All these results suggest that SRC3 is utilized in a gene and tissue specific manner by TR during development. It would be of considerable interest in the future to determine whether and how the promoter context and/or cofactor compositions in different tissues dictate the use of cofactors in gene regulation by TR in vivo.

Next, to determine whether SRC/p300 complex was indeed required, the in vivo role of the histone acetyltransferase p300/CREB binding protein (CBP) was also analyzed. ChIP assay revealed that p300 is recruited to TH responsive enhancers with a pattern similar to the one observed for SRC3. Furthermore, transgenic tadpoles overexpressing a dominant negative form of p300 (dnp300), containing only the SRC interacting domain, displayed arrested or delayed metamorphosis (Fig. 2) [78]. The molecular analyses of these animals showed the recruitment of the p300 mutant by TR, displacing endogenous p300 and leading to inhibition of TH responsive gene expression. These results thus suggest that p300 and/or its related CBP is an essential component of the TR signaling pathway in vivo. They also support the notion that p300/CBP and SRC3 proteins are part of the same complex regulating metamorphosis.

The SRC3/p300 coactivator complex is also composed of other proteins, including the coactivator associated arginine methyltransferase 1 (CARM1), which methylates arginine at positions 2, 17 and 26 of histone H3. *X. laevis* CARM1 was cloned and two alternative splicing forms, CARM1a and CARM1b, were obtained [79]. Both isoforms are expressed throughout metamorphosis. However, CARM1b had little effect on TR mediated transcription, whereas CARM1a enhanced gene activation by liganded TR. ChIP assays showed that both isoforms were recruited to the promoter by liganded TR. However, the binding of liganded TR to DNA was reduced with CARM1b, leading to a slight reduction in histone methylation at the TRE locus. These results suggest that CARM1 may play a role in the TR-mediated transcriptional regulation during metamorphosis and that its function is regulated by alternative splicing [79].

CARM1 is not the only arginine methyltransferase in the SRC3/p300 complex. Protein arginine methyltransferase 1 (PRMT1) acts as a transcription coactivator for nuclear receptors through histone H4 R3 methylation. The involvement of PRMT1 in TR function during amphibian development has also been analyzed [80]. PRMT1 expression was upregulated during metamorphosis. PRMT1 was recruited to TREs of endogenous target genes and enhanced TR binding to DNA. The ChIP data showing more TR binding in the presence of PRMT1 was most likely due to a more stable complex of TR with DNA and with coactivators (more coactivator would drive more complex formation), and not that liganded TR has more affinity of DNA. Another possibility could be that TR was more efficiently crosslinked to the DNA in the presence of PRMT1. The fact that PRMT1 also enhanced transcription would support the first explanation. PRMT1 recruitment thus led to transcriptional activation by liganded TR. Transgenic animals overexpressing PRMT1 showed an increase of TH target gene activation in vivo and accelerated natural and TH induced metamorphosis (Fig. 2). Surprisingly, PRMT1 was only transiently recruited to the TRE during metamorphosis. These results thus indicate that PRMT1 action as a coactivator in TR mediated transcription was transient to enhance TR binding to TRE and target gene activation [80]. These results further suggest that PRMT1 has tissue-specific roles in regulating the rate of metamorphosis.

Two other categories of multi protein coactivator complexes that also mediate TR activation of transcription in amphibians have been identified [76]. These are the mediator complexes such as vitamin D receptor interacting protein (DRIP), TR associated protein (TRAP), and activator recruited cofactor (ARC) complexes and chromatin remodeling complexes such as SWI/SNF. However, their role and function during development in vivo remains to be elucidated. Only two coactivators participating in such complexes were analyzed in the context of metamorphosis [81]. In this study, the expression of Brahma related gene 1 (BRG1), a chromatin remodeling enzyme, was found to be increased by T_3 at metamorphic climax in *X. laevis*, whereas BRG1 associated factor 57 (BAF57), a BRG1 binding protein member of the BRG1 containing chromatin remodeling complexes, was found to be expressed constantly during development. BRG1 and BAF57 are recruited by liganded TR to enhance the transcriptional activation. The effect relies on chromatin remodeling because a remodeling-defective BRG1 mutant inhibited the activation.

Thus multiple cofactor complexes have been identified which contribute to the transcriptional activation regulated by amphibian TRs. While they may have different and multiple roles in regulating transcription, one of their main actions is likely through chromatin modification.

4. Chromatin remodeling during gene regulation by TR

Wrapping DNA around the core histones in a nucleosome, the basic building block of chromatin, creates severe steric impediments for transcription factors that need to gain access to specific recognition sequences [82]. Chromatin is a dynamic structure that can be reorganized by corepressors or coactivators, thereby altering access of transcriptional machinery to the DNA. In addition to histones, genomic DNA in eukaryotic cells is also associated with other nuclear proteins, including transcription factors and cofactors. Transcriptionally active DNA has a protein composition different from transcriptionally silent DNA. There are two general types of chromatin reorganization/remodeling that can be distinguished: histone modifications [83,84] and chromatin structure alterations [85,86]. Both may affect DNA accessibility. Histone modifications are mostly post-translational modifications of the histone NH_2 terminal tail. At least 130 histone modifications have been reported [87], including acetylation, phosphorylation, methylation, propionylation, nutyrylation, formylation, ubiquitylation, sumoylation, citrullination, proline isomerization, ADP ribosylation, tyrosine hydroxylation and crotonylation. Chromatin structure alterations may involve changes in nucleosome structure, histone composition, and nucleosome removal [88,89]. Chromatin can have multiple roles in the regulation of TH target gene expression in vivo.

4.1. Chromatin structure alteration

Several studies have provided strong evidence that liganded TR induces extensive chromatin remodeling in vivo. First, analysis in the reconstituted frog oocyte system in vivo suggests that TR/RXR heterodimer is able to recognize a TRE within chromatin. This is probably because TR/RXR can bind to a TRE in vivo in the absence of TH. In addition, in the reconstituted frog oocyte system, where one can study gene regulation by TR in the context of chromatin, TR makes use of chromatin assembly processes to silence transcription efficiently and TR directs the disruption of local chromatin structure in response to TH [26,90–93]. These studies have correlated hormone-dependent transcriptional activation with chromatin remodeling (Fig. 3), often revealed by loss of canonical nucleosomal ladder, formation of DNase I hypersensitive sites, increased nuclease accessibility or changes in DNA topology. The disruption corresponds to a change that is equivalent to the loss of a few nucleosomes (2 to 3) per TR/RXR binding [90,91], a phenomenon that has also recently been

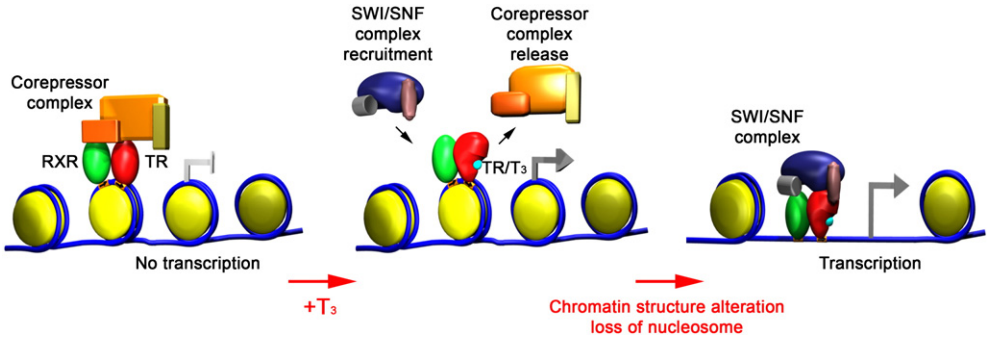

Fig. 3. Transcriptional regulation by TR through chromatin remodeling. In the absence of TH (left panel), the TR/RXR heterodimer binds to TREs (arrows on blue line representing the DNA around the histone octamers which appear as yellow cylinders). Then, the unliganded TR recruits the corepressor complex, which in turn causes chromatin to switch to a non-permissive state for transcription. This remodeling is dependent of HDACs and certain HMT activities and results in target genes silencing. Upon T_3 binding to TR (middle panel), conformational changes drive the release of the corepressor complex and the recruitment of the chromatin remodeling complex SWI/SNF. Such coactivator activities result in chromatin structure alteration and nucleosome loss (right panel), which causes chromatin to switch to a state permissive for transcription.

observed during metamorphosis. TH induces nucleosome removal on the endogenous TH response genes in the intestine of *X. tropicalis* tadpoles [94]. This was determined on endogenous TR target genes by carrying out ChIP assays with antibodies against the total H2B and H3 of the core histones on intestine isolated from premetamorphic tadpoles. Upon T_3 treatment of premetamorphic tadpoles, the amount of histones H2B and H3 was drastically reduced at the TRE regions of TH response genes. Conversely, a few changes were observed where there were no TRE sites, indicating specific nucleosome removal associated with gene activation by liganded TR during intestinal remodeling. This is likely the manifestation of the chromatin disruption observed in the frog oocyte transcription system.

The mechanism of this nucleosome loss remains to be determined. Given the presence of multiple chromatin remodeling factors, it has been difficult to pinpoint the specific effect and function of a single factor. However, studies suggest that this remodeling may be caused by the recruitment of BRG1 (SWI/SNF) chromatin remodeling complexes by liganded TR to the minichromosome in oocyte [76,81] as well as the recruitment of BAF57 [81]. SWI/SNF can alter chromatin structure by inducing changes in conformation and/or position (sliding), DNA topology or even the loss of histones/nucleosomes, which are likely required for exposing important regulatory DNA sequences to transcription factors and especially basal transcription machinery. Both BRG1 and BAF57 are expressed during intestinal metamorphosis in *X. laevis* [81]. It is very likely that liganded TR bound to the TREs of TH response genes in the tadpoles recruits BRG1 and BAF57 containing chromatin remodeling complexes to remove the nucleosomes at the target genes (Fig. 3).

Although previous studies suggest that nuclear receptors may recruit the SWI/SNF directly, SWI/SNF is unlikely to be recruited through a direct interaction with liganded TR. In oocytes, the recruitment of SWI/SNF is likely to be initiated through a direct or indirect interaction with CBP/p300, which in turn can modify histones (see below) to subsequently provide additional anchors that further stabilize the recruitment of SWI/SNF [76].

4.2. Histone modifications: acetylation

Many histone modifications are involved in the control of transcriptional regulation [95,96]. Some of them have been studied during metamorphosis (Fig. 4). First, acetylation allows neutralization of positive charges abundant in histone tails and reduces the affinity of the NH_2 terminal tails for negatively charged DNA. This change affects nucleosome conformation, enabling a more open chromatin that is more accessible to transcriptional machinery and favors the recruitment of other coactivators such as complexes containing SWI/SNF and mediator [76]. Histones H3 and H4 are deacetylated around the genomic loci containing a TRE in the absence of TH in tadpoles before metamorphosis. T_3 treatment increased histone H4 acetylation in vivo [35]. The levels of histone H3 and H4 acetylation were correlated with the levels of gene expression, TR binding and RNA polymerase II recruitment [97,94]. Treatment with trichostatin A (TSA), a HDAC inhibitor [98] caused an increase of histone H4 acetylation at TH response gene TREs and increased transcription [35,99,100], further supporting a role for histone acetylation in gene regulation by TR.

Five lysines can be acetylated in the NH2 terminal tail of histone H4. It has been shown that lysine 5 (H4K5) of H4 is the preferred target of HDAC3, although all lysines can be targeted by HDAC3 [101]. Consistently, T_3 treatment increased acetylation mainly at H4K5 [LM Sachs, unpublished data], likely due to the release of NCoR/HDAC3 or SMRT/HDAC3 corepressor complexes. Histone H3 also has five lysines that can be acetylated. High levels of AcH3K9 were detected in the absence of TH. The absence of any effect by TH on AcH3K9 correlates with the absence of SRC3 acetylation activity when recruited by

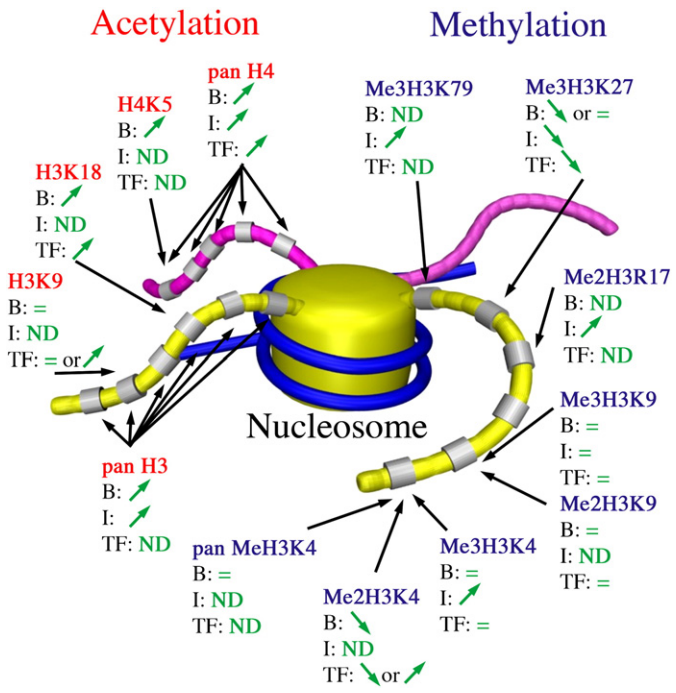

Fig. 4. Post-translational modifications of histone tails induced by T_3 during metamorphosis. During tadpole development, acetylation or methylation of the histone tail residues is both stage and tissue specific. Affected residues are mainly lysine (K) or arginine (R). Brain (B), Intestine (I) and Tail-fin (TF). Up arrows (↗), down arrows (↘), equal signs (=) and (ND) respectively correspond to an increase of, a decrease of, no change in, or no data available for the presence of the indicated mark in the specified context.

liganded TR. SRC3 is supposed to incorporate this mark. However, some data indicate that when SRC3 is recruited by a nuclear receptor, H3K9 acetylation activity is not required. Moreover, as AcH3K9 level is already high, recruitment of additional HAT may not matter. It has been shown that T_3 treatment increased lysine 18 (H3K18) acetylation [97]. This may be due to the recruitment of the acetylase p300, which can acetylate H4K5 and H3K18 [76]. Furthermore, it is generally believed that histone acetylation likely affects nucleosomal higher order chromatin structure, alters histone tail/DNA interaction and/or serves as a "code" for regulatory proteins (such as SWI/SNF) and basal transcriptional machinery docking. The histone acetylation exerted by CBP/p300 subsequently provides additional anchors to further stabilize the recruitment of SWI/SNF [76].

TR mutant overexpression and coregulators mutant analysis studies also highlight the importance of acetylation in gene regulation. The binding of dpTR to known TH target genes increased local histone acetylation and gene activation, similar to liganded TR during natural metamorphosis [48]. Alternatively, the transgenic overexpression of dnTR, which binds endogenous target promoters to repress their expression, led to retention of corepressors and continued histone deacetylation in the presence of TH. Likewise, transgenic overexpression of a dominant negative SRC3 [75], whose recruitment is TH dependent, blocks the recruitment of endogenous coactivators and histone acetylation and thereby preventing the TH dependent gene activation.

4.3. Histone modifications: methylation

Methylation represents a more complex type of modification that targets principally H3. Four arginine residues (R2, R8, R17 and R26) and six major lysine residues (K4, K9, K27, K36, K79 and K20) can each be mono, di or trimethylated. The position and the degree of methylation can have different transcriptional consequences, unlike histone lysine acetylation, which is generally associated with activation [102]. To determine whether these modifications play a role in gene regulation by TR during development, ChIP assays were carried out with antibodies against different histone modifications in several tissues (brain, intestine and/or tail fin) in premetamorphic tadpole and during TH induced or natural metamorphosis (Fig. 4).

First, H3 lysine 9 (H3K9) dimethylation and trimethylation, which are associated with closed chromatin and gene repression [103], were shown not to be involved in TR mediated T_3 gene regulation [97,94]. The other repressive mark [104], the trimethylation of histone H3 at lysine 27 (Me3H3K27), was tissue specific and, as expected inversely correlated with the basal level of TH response gene expression measured in the absence of TH. Then, T_3 treatment decreased the level of Me3H3K27 in proportion to the initial level of occupancy and initiated the transcriptional induction of T_3 target genes [97,94]. Thus, of the two repressive histone marks found in mammalian cells, only one, Me3H3K27, was correlated with gene regulation by TR during metamorphosis. Moreover, the histone methyltransferase (HMT) involved in Me3H3K27 acquisition is the protein enhancer of zeste 2 (ezh2), a core component of the Polycomb repressive complex 2 [105]. Utx and JMJD3 are the histone demethylases involved in removing the Me3H3K27 mark [106]. These results point to a novel role of Polycomb and Thrithorax for regulating TH response genes. Thus, a tissue specific recruitment of Polycomb complexes in the absence of T_3 may result in methylation of H3K27 and stronger gene repression. TH induced gene activation may involve the recruitment of Trithorax complexes to remove H3K27 methyl marks.

Next, H3 lysine 79 trimethylation (Me3H3K79) and H3 arginine 17 asymmetrically dimethylated (Me2H3R17), two histone marks that have clearly been associated with activation of gene transcription, were also analyzed on intestine isolated from premetamorphic tadpoles. The results showed that the levels of all these activation marks were increased upon T_3 treatment at the TRE regions [94], suggesting that the recruitment of DOT1 methylates H3K79 [107] and that CARM1 is recruited for arginine methylation [108,109]. However, no evidence has been found that DOT1 is in a coactivator complex recruited by TR. As previously indicated, CARM1 is part of such a coactivator complex [79]. Thus, these histone marks are associated with high levels of gene expression in mammalian cells and utilized by TR for transcriptional activation of target genes in amphibians.

Finally, H3 methylation at lysine 4 (MeH3K4), which can correlate with either activation or repression [96], has TH dependent gene and tissue specific variations in premetamorphic tadpoles following T_3 treatment. T_3 decreased levels of dimethyl H3K4 (Me2H3K4) on one of the TRE locus and increased it on another TR binding site [97]. Similarly, depending on the TRE analyzed, trimethyl H3K4 (Me3H3K4) was clearly present with no effect of T_3, increased following T_3 treatment or was absent to low level depending the tissues analyzed [97,94]. The results showed that at the TRE regions of TH activated genes, the levels of global H3K4 methylation (Me2 and Me3) were increased upon T_3 treatment. This is in agreement with the correlation between these marks and gene activation observed in mammals. To further address the role of H3K4 methylation, the effect of a HDM inhibitor involved in the deposition of this mark was investigated [97]. Pargylin is a monoamine oxidase inhibitor that blocks LSD1 [110], the first HDM described [111]. LSD1 was shown to demethylate mono and dimethyl H3K4. Pargylin treatment increased TH response gene expression and H4 acetylation indicating that HDM requirement is part of the TR regulation processes. The increase in AcH4 strongly emphasizes the functional interplay between demethylation and deacetylation. Furthermore, H3K4 methylation levels correlated interestingly, with TR binding to TRE [97]. Again, making use of Pargylin, the HDM inhibitor increased H3K4 methylation at the TRE region and significantly increased TR binding to DNA at the same locus. The present data thus demonstrates a strong link between H3K4 methylation and TR binding. This observation is in accordance with the proposed function of H3K4 methylation mark and the observations from whole genome mapping of H3K4 methylation, which showed strong association with enhancer or promoter regions [112,113].

Interestingly, even though Me3H3K27 and Me3H3K4 are considered to have opposing effects on gene activity, these studies suggested Me3H3K27 and Me3H3K4 are simultaneously recruited to the same TRE regions [114]. They could be concomitant on a certain DNA locus with the repression function of Me3H3K27 having a dominating effect. Most of these bivalent genes are either poised for transcription, with preinitiated RNA Polymerase II at their promoter [115] or transcribed at a low level [116]. Such a bivalent situation has also been observed for TH response genes [97] but clear demonstration has not been obtained yet. It is possible that the Me3H3K27 and Me3H3K4 do not occur at the same site. However, cobinding of HDAC1 and p300 at the enhancer of the same silent locus was observed for many bivalent genes [116] and for some TREs [47].

5. Model of thyroid hormone receptor mechanism of action

Over a decade ago, a working model was proposed to explain the mechanism of repression by unliganded TR and activation of transcription by liganded TR on positively regulated genes [117]. It is still largely valid today but should be modified into a more complex model, reflecting the knowledge accumulated from amphibian studies. These studies demonstrated that TREs should be organized into two categories. The first one is linked to TREs that bind unliganded and liganded TR (Fig. 5A and B) as found on the *TRβ* gene locus, and the second one to TREs that only bind liganded TR, as identified on the *TH/bZIP* gene locus in tail fin (Fig. 5C and D) [97]. The TRE locus seems first to be devoid of H3K9 methylation marks which involve the action of H3K9 HDM or/and the inhibition of H3K9 HMT before and in concomitance with TR binding.

In the absence of T_3, TR and RXR bind to TREs assembled in chromatin containing H3K4 methylation (compare Fig. 5A to C). High level of H3K4 methylation correlates with high level of TR binding to DNA while low level is associated with absent to weak binding. The deposition of H3K4 methylation requires the recruitment of Me2H3K4 and Me3H3K4 HMT. Conversely, the absence of H3K4 methylation requires the recruitment of Me2H3K4 and Me3H3K4 HDM. The heterodimer also recruits an NCoR/SMRT corepressor complex that contains at least HDAC activity (Fig. 5A and C). Data strongly suggest a unique role of NCoR/HDAC3 in repression by unliganded TR. However, it is possible that HDAC1 contributes to TR repression in other cell types or other genes as shown in *X. laevis*. Histone deacetylation leads to a closed chromatin conformation, inaccessible for transcriptional machinery. The repressed TH response gene is also associated with high level of Me3H3K27 that is deposited by ezh2 (Me3H3K27 HMT), the catalytic subunit of polycomb repressive complex. Other effects involving chromatin remodeling or direct interaction with transcriptional machinery could be involved. In mammals, some components of the SWI/SNF chromatin remodeling complexes were copurified with NCoR [118].

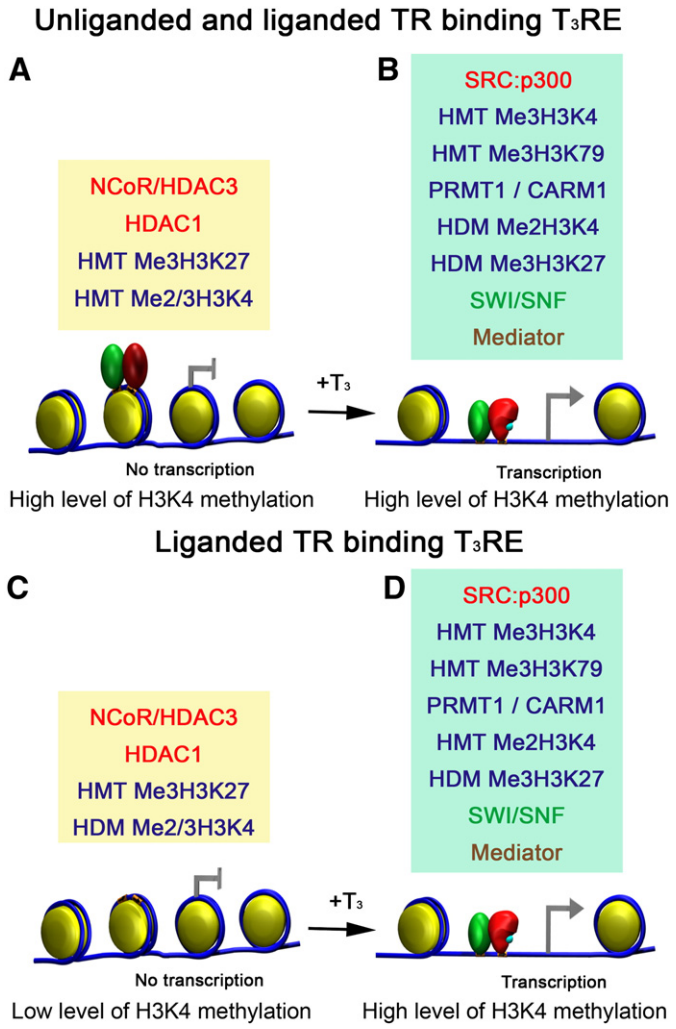

Fig. 5. Mechanisms of thyroid hormone receptor action in vivo. In the absence of ligand, direct response genes are silenced by two mechanisms, depending on the methylation state of the chromatin at TREs. First, TREs enriched in H3K4 methylation marks following the action of specific H3K4 HMTs can be bound by unliganded TR/RXR (upper left panel). In contrast, TREs not enriched in H3K4 methylation marks, a chromatin modification mark deposited by specific HDMs (HDM Me2/3 H3K4 such as lsd1), are not bound by unliganded TR/RXR (lower left panel). However, regardless of the presence or absence of TR, NCoR appears to be present at these genes to keep the chromatin in a non-permissive state for transcription, mainly through the action of HDAC3. These genes are also associated with high levels of Me3H3K27, a chromatin modification mark deposited by Me3H3K27 HMT such as EZH2, and strongly associated with gene silencing. Upon T_3 binding to TR, conformational changes occur for TR/RXR both already bound to TREs (upper right panel) or free in the nucleus, enabling the latter to bind at TREs depleted in H3K4 methylation marks (lower right panel). Liganded TR recruits coactivator complexes, which induces transcription-permissive chromatin state. In particular, histone acetylation of lysine residues is likely to be carried out by p300 or related CBP, while both methylation and demethylation involve distinct HDMs and HMTs.

T_3 binding induces a conformational change of TR that relieves its inhibitory effect when already present on TRE loci (Fig. 5A toward 5B) and increases TR binding at TRE loci containing low levels of H3K4 (Fig. 5C toward D). Liganded TR will induce two changes: the release of the NCoR/HDAC3 corepressor complex followed by the recruitment of a coactivator complex such as SRC/p300 (Fig. 5C toward D). The HAT activity, specifically carried by p300 in this coactivator complex, leads to the acetylation of several histone H3 and H4 lysine residues (at least H4K5 and H3K18). Interestingly, for some TREs, HDAC1 is sequestered in a transcriptional activation context (Fig. 5B) while in other TREs it is released as expected (Fig. 5D). The gene specific function of such phenomenon is not understood but, as previously indicated in mammals, whole genome analysis of HAT and HDAC recruitment has led to the conclusion that both activities are required for gene activation [71]. Methyltransferases, such as CARM1 (H3R17) and PRMT1 (H4R3) are also recruited into the SRC/p300 complex (Fig. 5B and D).

The modification of histone methylation profiles is required for TH induced transcriptional activation. The H3K27 methylation first has to be removed because this repressive mark exerts a dominant effect on the other marks. This is made evident by the decreases in Me3H3K27 levels on the TRE locus upon treatment with T_3, strongly suggesting the recruitment of Me3H3K27 HDM (Fig. 5B and D). Second, the H3K79 methylation level increases at the liganded TR bound to DNA loci. This mark, associated with gene activation, has to be deposited by a H3K79 HMT such as Dot1 (Fig. 5B and D). Third, a more complex H3K4 methylation pattern is observed that is gene and tissue specific. On the one hand, when the Me2H3K4 and Me3H3K4 levels were high in the absence of TH (Fig. 5A), the liganded TR led to the decrease of Me2H3K4 level and had no effect on Me3H3K4 level, which suggests the presence of Me2H3K4 HDM and Me3H3K4 HMT (Fig. 5B). On the other hand, when the Me2H3K4 and Me3H3K4 levels were low in the absence of TH (Fig. 5C), the liganded TR led to the increase of Me2H3K4 level and had no effect on Me3H3K4 level, which suggests the presence of Me2H3K4 HMT (Fig. 5D). However, in some tissues, an increase of Me3H3K4 level is measured suggesting also the recruitment of a Me3H3K4 HMT. The H3K4 residue is thus methylated in correlation with the transcriptional activity of the TH response gene.

All histone modifications lead to chromatin opening but other processes, such as nucleosome excision and transcriptional machinery recruitment, promote transcription activation. Upon T_3 treatment, TR can also interact with coactivator complexes such as the SWI/SNF complex involved in chromatin remodeling and the mediator complex directly involved in transcription activation. Although it seems unlikely that any single core promoter requires all the transcription complexes and different complexes are likely required for regulating distinct *cis* DNA elements in a temporal and tissue specific manner, it is also possible that these complexes have different functions and TR recruits all of them. If the later is the case, the histone acetylation by SRC/p300 complex could be a prerequisite for the SWI/SNF and mediator complex recruitment and functions at specific TH response promoters [76]. Evidence therefore suggests that for TH dependent genes, TR coactivators can be recruited through a direct or indirect effect: TR/coactivator, coactivator/coactivator or coactivator/histone interaction. In such a dynamic process, liganded TR first interacts with the SRC/p300 complex. Then, p300 recruits SWI/SNF through direct or indirect interaction and its histone acetyltransferase

activity is required to further stabilize this interaction. Finally, the mediator complex is recruited through direct interaction with TR or indirectly through p300. Thus, the model proposed indicates that multiple cofactors carrying various histone modifications, chromatin structure modifying and transcriptional machinery recruitment activities are required for gene activation as well as gene repression.

6. Conclusions and perspectives

Anuran metamorphosis serves as an excellent model to understand the molecular mechanism of transcriptional regulation by TR in vivo during postembryonic development in vertebrates. Studies have provided strong evidence to show that TR has dual functions during development: first as an unliganded repressor of gene expression to ensure a proper tadpole growth period, and later, as a liganded activator to induce metamorphosis. These dual roles are exerted by recruiting corepressors and coactivators, respectively. While this basic dual function model is well supported, the complex details of the molecular pathways are just starting to be understood. Future work should focus on the regulation and effects of histone modifications, cofactor recruitment and dynamics of these histone modifications deposition and coregulator recruitment. Finally, metamorphosis also provides a wonderful system to address the tissue and gene specificity of TR action. High throughput technology will allow a better understanding of whole genome effects [119]. Furthermore, the genome is a complex regulatory landscape where genome topology is expected to cause variation in the transcriptome [120]. Three dimensional genome maps are required to understand all these regulatory networks.

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