

# What is the Role of Thyroid Hormone Receptor Alpha 2 (TR $\alpha$ 2) in Human Physiology?

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## ABSTRACT

Thyroid hormone receptors are nuclear receptors that function as transcription factors and are regulated by thyroid hormones. To date, a number of variants and isoforms are known. This review focuses on the thyroid hormone receptor  $\alpha$  (TR $\alpha$ ), in particular TR $\alpha$ 2, an isoform that arises from alternative splicing of the *THRA* mRNA transcript. Unlike the TR $\alpha$ 1 isoform, which can bind T3, the TR $\alpha$ 2 isoform lacks a ligand-binding domain but still binds to DNA thereby antagonizing the transcriptional activity of TR $\alpha$ 1. Although a regulatory role has been proposed, the physiological function of this TR $\alpha$ 2 antagonism is still unclear due to limited *in vitro* and mouse model data. Recently, the first patients with resistance to thyroid hormone due to mutations in *THRA*, the TR $\alpha$  encoding gene, affecting the antagonistic function of TR $\alpha$ 2 were described, suggesting a significant role of this particular isoform in human physiology.

The thyroid hormones (TH) triiodothyronine (T3) and thyroxine (T4) are important regulators of biological functions. Lack of TH action can lead to developmental, metabolic, and cardiovascular diseases. At the cellular level, nuclear receptors called “thyroid hormone receptors” (THRs) mediate TH function [1]. Heritable syndromes of impaired TH sensitivity include, among others, resistance to thyroid hormone (RTH), which is caused by mutations in genes encoding for THRs, in particular the two genes *THRA* and *THRB* [2]. In general, patients’ symptoms depend on the expression pattern of the affected gene and the resulting functional defect. Refetoff et al. coined the acronym RTH when they described the

first patient in 1967 [3], who, 20 years later, was found to have a homozygous deletion in the *THRB* gene encoding TR $\beta$  [4]. During this time, additional patients with RTH were identified, most of whom carried a heterozygous missense mutation in *THRB* [5, 6]. Patients with RTH have elevated T3 and T4 serum concentrations, with some having elevated, but never suppressed thyrotropin (TSH) levels, making the association with TH-dependent disease relatively clear. The phenotype is variable, and few patients show severe symptoms such as attention-deficit hyperactivity disorder (ADHD), tachycardia, or goiter [7, 8]. TR $\beta$  is mainly responsible for the negative feedback loop regulating the hypothalamus-pituitary-thyroid

axis [9, 10], which explains why TSH is not suppressed despite high T3 and T4 levels.

The two genes encoding the TH receptors are TR $\alpha$  (*THRA*) and TR $\beta$  (*THRB*), therefore, the differences in the phenotype of patients with TH receptor gene mutations are likely due to different expression patterns of the two isoforms. With the development of new technologies such as whole-exome sequencing (WES), the first *THRA* mutation was identified in 2012 [11], followed shortly by a second case in the same year identified by Sanger sequencing [12]. Since then, the number of *THRA* missense mutations has been steadily increasing. These patients lack the changes in blood TH concentration that make other conditions with RTH so distinct, as the hypothalamus-pituitary-thyroid axis is only regulated by TR $\beta$ , whereas TR $\alpha$  plays no role in this feedback loop. Therefore, the T3 and T4 serum levels are mainly normal in these patients. It appears that the T3 levels are in the higher normal range while T4 is rather low-normal resulting in a shifted T3/T4 ratio; however, so far, no reference values are available for the T3/4 ratio leaving the relevance of this shift open. The normal T3 and T4 values in most cases make the phenotype even more striking, as *THRA* mutation carriers have an even more severe phenotype than *TRHB* mutation carriers with mainly hypothyroid symptoms. These include growth retardation, mild to moderate mental retardation, mild skeletal dysplasia, severe constipation, broad facial features, and bradycardia. Interestingly, most of the identified mutations result in partial or complete loss of function that inhibits gene regulation in a dominant-negative manner [11–22].

Recently, we described a novel heterozygous point mutation that enhances the function of both TR $\alpha$  isoforms, TR $\alpha$ 1 and TR $\alpha$ 2 leading to increased T3-activation of TR $\alpha$ 1 and increased antagonism of TR $\alpha$ 2 [23]. This novel mechanism in RTH due to *THRA* mutations brings the TR $\alpha$ 2 splice variant into focus and raises further interest in its physiological function.

The TR $\alpha$  encoding gene *THRA* (17q21.1) was previously described as a proto-oncogene “c-erb-A” and was isolated from embryonic chicken, human placental, and rat brain libraries [24, 25]. This gene encodes proteins that share structural features with other nuclear receptors, consisting of a regulatory A/B-domain, a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) (► Fig. 1a) [26, 27]. C-erb-A was identified as TR $\alpha$ 1 by nuclear localization, ability to specifically bind TH and transcriptional regulation of TH-responsive genes [24, 25]. Briefly, TR $\alpha$ 1 can interact with TH responsive elements (TRE) located in the promoter regions of TH-regulated genes *via* the two C<sub>4</sub>-zinc fingers in the DBD and regulate transcription (► Fig. 2a) [28]. Even in an unliganded state, TRs occupy TREs in the function of transcriptional regulators [29].

TREs typically consist of a 5′-AGGTCA-3′ motif arranged in repeats, either as palindromic, inverted palindromic, or direct repeats spaced by four nucleotides (DR4) [30]. TRs can be positive or negative regulators being able to initiate or inhibit transcription depending on which TRE is bound [31]. The C-terminal LBD is crucial not only for regulating activity through ligand binding but also for interacting with cofactors and dimerizing with other nuclear receptors. Upon binding to T3, the LBD undergoes conformational changes that lead to the replacement of co-repressors (CoRs) by co-activators (CoAs) (► Fig. 2b) [32]. TRs are known to exist as monomers, but can also

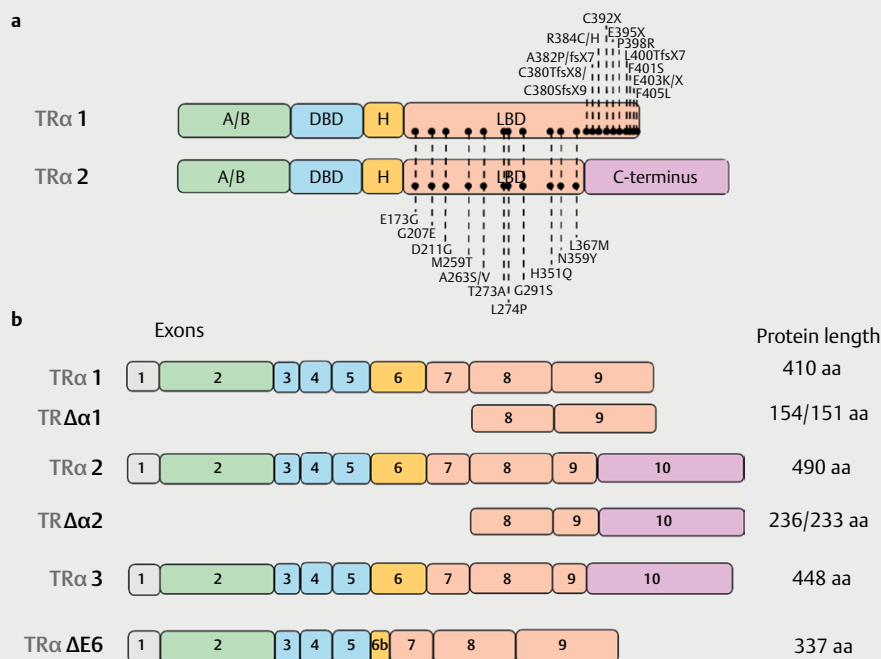
form homodimers [33] as well as heterodimers with other nuclear receptors, the most common being the retinoid X receptor (RXR) [34, 35]. Interestingly, RXR has been shown to significantly inhibit transcriptional activity *in vitro*, suggesting a regulatory role for this heterodimer [36].

In addition to the canonical function as transcription regulators, non-canonical TH signaling has a more rapid effect on the target cell. Flamant et al. proposed a classification of TH action into four subtypes [37]: **Type 1** corresponds to the canonical model of TR as a transcription factor by direct binding to DNA, as described above, with TH signaling in mitochondria *via* the shorter isoform (as described below) also belonging to this type. **Type 2** includes signaling *via* indirect binding to DNA, e. g., by binding to other transcription factors. **Type 3** includes signaling independent of DNA binding, such as direct activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, which is described in particular for the plasma membrane-bound isoform p30 (see below). **Type 4** summarizes TH signaling independent of TRs, e. g., integrin  $\alpha$ v $\beta$ 3, which has been proposed as a membrane receptor for T3 and T4.

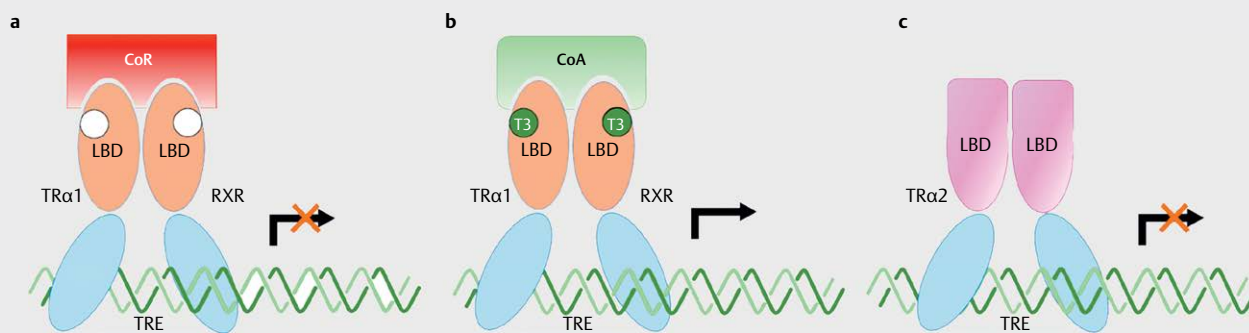
Shortly after the discovery of TR $\alpha$ 1, the TR $\alpha$ 2-isoform was identified resulting from an alternative splice site at exon 9 and transcription of an additional exon 10 [38, 39] (► Fig. 1b). Interestingly, this isoform is unable to bind TH due to the extended C-terminal LBD [40] leading to an antagonistic effect on TR $\alpha$ 1 and TR $\beta$ . However, TR $\alpha$ 2 has only a weak antagonistic effect on TR $\alpha$ 1 and TR $\beta$ , as it needs high expression levels to inhibit transcriptional activity (► Fig. 2c) [41–44] suggesting a rather minor physiological role for TR $\alpha$ 2. Several mechanisms have been suggested to explain this phenomenon: (i) competition for TRE binding sites, (ii) interaction with RXR as the preferred dimerization partner of TR $\alpha$ 1 on specific TREs such as DR4 [45], or (iii) a DNA-independent mechanism such as interaction with basal transcriptional factors [41, 46]. Moreover, the phosphorylation state of the elongated C-tail has been shown to influence the antagonistic effect of TR $\alpha$ 2 [47]. Interestingly, the inhibitory effect seems to occur only on positive TREs [48], and not on negative TREs. The rather weak effect can be explained by the lack of interaction between TR $\alpha$ 2 and CoRs [42, 43].

Nevertheless, protein studies in mouse models and *post mortem* human brains indicate a high expression ratio of TR $\alpha$ 2 to TR $\alpha$ 1 [49, 50]. This suggests a T3-independent regulatory role for the physiology of TR $\alpha$ 2. Important for this role is that TR $\alpha$ 2 has a functional DBD, which still binds to TREs as homo- or heterodimer with RXR, albeit with lower affinity as compared to TR $\alpha$ 1 [42, 45]. Therefore, as long as TR $\alpha$ 2 is highly expressed, it forms homodimers and occupies TREs without the capability to be activated by T3 and thus acts as an antagonist to TR $\alpha$ 1. A direct heterodimer with TR $\alpha$ 1, which would result in an even more direct TR $\alpha$ 1-antagonism, was not observed on any tested TRE. However, since most dimerization studies of TRs examined the formation of dimers on TREs indirectly by using DNA mobility shift assays, it cannot be excluded that heterodimerization between TR $\alpha$ 1 and TR $\alpha$ 2 may occur independently of DNA-binding, which was suggested by Katz et al. 1995 [47].

Over time, other TR $\alpha$  isoforms were discovered (► Fig. 1b), including a suspected third splicing variant, TR $\alpha$ 3 that originates from another splicing event in exon 9. Similar to TR $\alpha$ 2, this isoform presumably has an elongated C-tail encoded by the sequence of exon 10 (448 amino acids), which is also unable to bind to TH. As only one



► **Fig. 1** (a) Functional domains of TRα1 and TRα2 isoforms. Published disease-causing variants are marked on the protein domain structure. (b) TRα isoforms resulting from alternative splicing, or different transcription start points, leading to proteins of different molecular weight. (A/B: a regulatory domain; DBD: DNA-binding domain; LBD: ligand-binding domain)



► **Fig. 2** (a) The DNA binding domains (DBD) of the unliganded TRα1 homodimer bind to responsive elements of the thyroid hormone receptor (TRE) in the promoter region of target genes. The ligand-binding domains (LBD) are in complex with co-repressors (CoRs) and target gene expression is inhibited. (b) Upon binding of thyroid hormone T3, CoRs are exchanged for co-activators (CoA), and the target genes are then expressed. (c) The weak antagonistic effect of TRα2 is probably due to competition for TREs between both isoforms without interaction with CoRs.

study was able to detect this isoform, [38] it is vastly understudied, but the predicted structure anticipates the same function as TRα2. Yet another alternative splicing event is responsible for the TRα1-ΔE6 isoform that carries an exchange of exon 6 for the micro-exon 6b. The resulting protein lacks T3-binding capacity but can reduce the transcription-enhancing activity of TRα1. Its proposed role is also regulatory for TRα2 and seems to be important for myocardial development, though the expression pattern of TRα1-ΔE6 suggests additional roles in other tissues [51]. Although it has not been investigated, a TRα2-ΔE6 isoform likely exists as well.

Further truncated isoforms, ΔTRα1 and ΔTRα2, have been observed, which are the result of transcription from an internal promoter in intron 7 and thus are missing the N-terminal domain, but otherwise resemble TRα1 and TRα2 [52]. Additionally, alternative translational start points on the TRα1 mRNA can result in shorter isoforms (p43, p30, p33, and p28) that can be activated by T3 but lack the ability to bind to DNA. They are thought to be bound to the plasma membrane (p30 and p33) [53, 54] or located in the mitochondria (p28 and p43) [55] and maybe responsible for more immediate non-canonical signaling *via* the MAPK pathway.

Since the first description of a loss-of-function *THRA* mutation in 2012, about 26 other missense mutations have been identified. One-half of the identified mutations are positioned in the TR $\alpha$ 1-specific region of the LBD [11, 12, 14, 16, 19, 55–60], the other half is located in the coding exons that are shared between TR $\alpha$ 1 and TR $\alpha$ 2 [13, 15–22, 61] (► Fig. 1). The latter ones could also disturb the function of TR $\alpha$ 2, so the phenotypes seen in these patients might provide information about the physiological role of TR $\alpha$ 2.

Although several studies in mice have addressed TR $\alpha$ 1 and its function [62–66], the relative contributions of both isoforms to the overall phenotype have proven difficult to dissect. As mentioned earlier, a comparison of protein levels of TR isoforms in mice revealed organ-specific expression, particularly a unique expression pattern of high TR $\alpha$ 2 abundance in the central nervous system [49], suggesting an important regulatory role. The specific knockout of TR $\alpha$ 2 by adding a strong polyadenylation site that follows the stop codon of TR $\alpha$ 1 and transcriptional stop codon resulted in overexpression of TR $\alpha$ 1 and a mixed phenotype with hyper- and hypothyroid tissue states [67]. Although this model provides valuable information to the field, it could not fully explain the physiological role of TR $\alpha$ 2. Another interesting model is the *Pax8*<sup>-/-</sup>TR $\alpha$ <sup>0/0</sup> compound mouse. Here *Pax8*, a differentiation factor for thyroid cells was knocked out, which on its own results in the absence of thyroid cells and consequently the complete absence of TH. Without T4 treatment, this defect leads to an early death around weaning time [68]. This model was combined with a TR<sup>0/0</sup> model, harboring a complete deletion of all known TR $\alpha$  isoforms, which on its own was viable, but exhibited reduced growth, delayed bone maturation, moderate hyperthermia, and reduced intestinal mucosal thickness [69]. The *Pax8*<sup>-/-</sup>TR $\alpha$ <sup>0/0</sup> compound model survived without TH-treatment and partially rescued the lethal phenotype of *Pax8*<sup>-/-</sup> mice, but growth was delayed [70]. This study helped to understand how the unliganded receptors might have a physiological function and TH is required to relieve these effects during the postnatal stage. In contrast, *Pax8*<sup>-/-</sup>TR $\alpha$ 1<sup>-/-</sup> compound models, which still express TR $\alpha$ 2 and  $\Delta$ TR $\alpha$ 2 isoforms, have a similar lethal phenotype to *Pax8*<sup>-/-</sup> mice, probably due to an intact TR $\alpha$ 2 isoform that could affect the activity of other THR $\alpha$ s such as TR $\beta$  [71]. When comparing these two studies, a possible physiological role for TR $\alpha$ 2 is to modulate survival, especially in the first weeks of postnatal development.

Most mutations found in patients were studied *in vitro* using reporter gene assays based on TRE-dependent luciferase expression and interaction with DNA or with cofactors to show how they inhibit TR $\alpha$  function. For mutations jointly affecting TR $\alpha$ 1 and TR $\alpha$ 2, some but not all studies have also examined the effects in both splicing isoforms. However, when TR $\alpha$ 2 function was tested, most mutations had no measurable effect on antagonistic function. Interestingly, for two mutations (p.A263S and p.N359Y) the inhibitory effect of TR $\alpha$ 2 on TR $\alpha$ 1 was slightly reduced [15, 16], suggesting a decrease in the dominant-negative effect.

In contrast to all other studies, we recently reported a *THRA* mutation that resulted in a **gain-of-function** in both isoforms [23]. A mutated glutamate-to-glycine residue in the first helix of the LBD had a promoting effect on T3-inducible TR $\alpha$ 1 activity but also resulted in a gain-of-antagonistic effect for TR $\alpha$ 2. Based on the computational model of the LBD, we suspect altered dimerization

interphase, although an altered interaction with TRE or cofactors cannot be excluded. Nonetheless, this mutation is the first to enhance TR $\alpha$ 2 function by increasing its antagonistic capacity, at least *in vitro*. At the same time, TR $\alpha$ 1 function was increased as well, leading to a pronounced T3 effect. Given this strong gain of function effect of TR $\alpha$ 1 *in vitro*, one would expect a hyperthyroid phenotype of the patients, but this was only the case in patients with mild tachycardia. In fact, we observed more hypothyroid symptoms such as low IQ and global developmental delay, severe constipation, and obesity. Matching these symptoms with our *in vitro* results suggests that the activated antagonistic effect of the mutant TR $\alpha$ 2 was able to counteract the increased activity of the mutant TR $\alpha$ 1. Since in most brain regions the TR $\alpha$ 2:TR $\alpha$ 1 ratio is high [49, 50], the mutant TR $\alpha$ 2 appears to significantly suppress the activation of the mutant TR $\alpha$ 1, eventually leading to the neuronal hypothyroidism of patients with the THR $\alpha$ p.(E173G) mutation. In other tissues with predominant TR $\alpha$ 1 expression, such as cardiomyocytes, the gain-of-function mutation of TR $\alpha$ 1 without TR $\alpha$ 2 antagonism results in hyperthyroidism-like symptoms. These particular findings of the p.(E173G)-mutant, leading simultaneously to activation of TR $\alpha$ 1 and enhanced antagonism of TR $\alpha$ 2, suggests that the physiological function of TR $\alpha$ 2 is antagonistic to TR $\alpha$ 1 function, which appears to be important for the tissue-specific fine-tuning of TH action in target cells.

Overall, 33 years after the discovery of TR $\alpha$ 2 and almost 10 years after the first description of patients with mutations in *THRA*, the first evidence for a physiological effect of TR $\alpha$ 2 was found only recently in particular patients carrying a new TR $\alpha$ -mutation. So far, the finding is limited to a single case report and in principle other -potentially genetic- effects can influence the patient's wide phenotype. However, the obvious antagonistic effect of TR $\alpha$ 2, and its increase through this p.(E173G) mutation, proposes a novel mechanism in RTH due to *THRA* mutations and argues that TR $\alpha$ 2 indeed plays a role in controlling the local response of target cells to circulating T3. It is tempting to speculate that any mechanism that increases TR $\alpha$ 2 expression relative to TR $\alpha$ 1 will decrease the cell response to T3. Moreover, even in tissues with low T3 availability, high levels of TR $\alpha$ 2, or mechanisms that increase the DNA-binding of TR $\alpha$ 2, are more likely to suppress T3-responsive genes. TR $\alpha$ 2 was discovered in the 1980s but few publications on this isoform have appeared in recent decades, thus, it is now time to unravel the physiological role of TR $\alpha$ 2 at different developmental time points and in different tissues more thoroughly. Here, special attention must be paid to a clear distinction between the isoforms. Most likely, the potential of single-cell sequencing will stimulate this process and could lead to new and surprising discoveries for the other TR $\alpha$  isoforms that have been little studied so far. Unfortunately, the lack of suitable TR $\alpha$  antibodies, let alone isoform-specific antibodies, prevents the generation of genome-wide chromatin immunoprecipitation sequencing data (ChIP-Seq) of any species. For now, a lot of knowledge regarding TR $\alpha$  isoforms remains to be uncovered.

## Conflicts of Interest

The authors declare that they had no conflict of interest.

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