Title**: RECEPTORS of THYROID HORMONES**
Running title*:* **Receptors of Thyroid Hormones**

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Abstract
The important physiological actions of the thyroid hormones are mediated by binding to nuclear thyroid hormone receptors (TRs), encoded by two genes TRα and TRβ. These receptors act as hormone-dependent transcription factors by binding to DNA motifs located in the regulatory regions of target genes and recruiting coregulators (coactivators and corepresors), which alter chromatin structure. Novel thyromimetics have been developed that bind preferentially TRβ and could be used for treatment of hyperlipidemia and obesity. TRβ gene mutations cause resistance to thyroid hormones (RTH), characterized by unappropriately high thyroid-stimulating hormone (TSH) levels due to lack of feedback inhibition of thyroid hormones on the hypothalamus and pituitary gland, and to reduced sensitivity of other TRβ target tissues to thyroid hormones. Very recently, patients heterozygous for TRα mutations have been identified. These patients exhibit clinical symptoms of hypothyroidism in TRα target tissues such as intestine or hearth and near normal circulating TSH and thyroid hormone levels.

Thyroid hormone synthesis and metabolism

The thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), play an essential role in growth, development, and metabolism. THs are synthetized in the follicles of the thyroid gland by iodination of tyrosine residues of the thyroid-specific protein thyroglobulin (Rubio and Medeiros-Neto 2009). Thyroid hormone synthesis is stimulated by the thyroid-stimulating hormone (TSH), which is secreted by the thyrotropes of the anterior pituitary gland. In turn, TSH release is stimulated by the thyrotropin-releasing hormone (TRH), produced by the hypothalamic neurons of the paraventricular nucleus. Under normal conditions a feedback mechanism in which circulating thyroid hormones repress TSH and TRH gene transcription ensures the production of appropriate amounts of thyroid hormones by the thyroid gland.

THs are released by the thyroid gland to the bloodstream and they enter the cells through the ATP-dependent monocarboxylate transporters MCT8 and MCT10 and the organic anion transporter proteins (OATPs) (Visser *et al*. 2011). The gene encoding MCT8 is located on the X chromosome, and hemizygous

MCT8 mutations in males cause the Allan-Herndon-Dudley syndrome, characterized by abnormal serum TH levels and severe psychomotor retardation (Dumitrescu *et al*. 2004).

TH actions are mediated by binding to the nuclear thyroid hormone receptors (TRs) (Flamant *et al*. 2006; Brent 2012). Although the thyroid gland produces predominantly T4, T3 is the most active hormone, since it has a higher affinity by TRs. The amount of T3 available for binding to the nuclear receptors is regulated by the action of selenoenzymes deiodinases (DIOs). DIO1 and DIO2 catalyze the conversion of T4 to T3 in target tissues increasing intracellular levels of the active hormone, while DIO3 causes hormone inactivation since it converts T4 and T3 by inner ring deiodination to the inactive metabolites reverse T3 (rT3) and T2, respectively. Mutations in the *SECISBP2* gene, which is required for the synthesis of selenoproteins including TH deiodinases, alter the intracellular metabolism of TH and affected patients show short stature and delayed bone age (Dumitrescu *et al*. 2005).

Mechanism of Action of THs

TRs belong to the superfamily of nuclear receptors and act as ligand-dependent transcription factors. Besides TRs, the nuclear receptors superfamily includes the receptors for steroid hormones, lipophilic vitamins, cholesterol derivatives, and other metabolites, as well as "orphan" receptors with unknown ligands (Aranda and Pascual 2001).

TRs, as well as other non-steroid receptors, even in the absence of hormone are preferentially located in the nucleus and can interact directly with chromatin (Figure 1). TRs regulate transcription by binding to DNA sequences termed positive or negative hormone response elements (TREs), normally located in regulatory regions of target genes. The effects of the receptors on transcription are mediated through recruitment of coregulators. TRs can bind corepressors and actively repress target gene expression in the absence of ligand. Upon hormone binding the receptors undergo a conformational change that causes corepressor release and the recruitment of coactivator complexes and transcriptional activation of genes containing TREs. TRs can also regulate expression of genes that do not contain TREs by modulation of the activity of signaling pathways and transcription factors that bind to the target promoter, and THs can also elicit rapid responses also called "non genomic" or "non-genotropic" which are not blocked by inhibitors of transcription or translation. These rapid actions could be mediated by a fraction of membrane-associated nuclear receptors, or by occupancy of a putative membrane receptor coupled through appropriate second-messenger systems to the generation of the biological response. Through these "non-genomic" mechanisms THs can increase intracellular calcium or activate mitogen-activated protein kinases (MAPKs) or phosphoinositol-3-kinase (PI3K) to provoke cellular effects (Cheng *et al*. 2010).

The thyroid hormone nuclear receptor isoforms

TRs are encoded by two genes (α and β). Several TR protein isoforms are generated from the primary transcripts of the *TR*α and *TR*β genes (Figure 2), which are located in human chromosomes 17 and 3, respectively. The α1 and α2 isoforms are produced by alternative splicing of the *TR*α gene and differ at the carboxy-terminus. Whereas TRα1 is a bona fide receptor, TRα2 cannot

bind hormone with high affinity and therefore does not function as a receptor, having an antagonistic effect on TR actions. By usage of different transcription start points the *TRβ* gene gives rise to two main isoforms β1 and β2, with different amino terminus but capable of DNA and hormone binding. Other minor TRβ isoform TRβ3, as well as truncated forms with dominant negative activity (ΔTRβ3, ΔTRα1 and ΔTRα2) which cannot bind DNA, have been also identified in rodents although their functions have not yet been defined (Refetoff and Dumitrescu 2007)

TRα1, TRβ1 and TRβ2 are the main hormone-binding isoforms and share a common structure with other nuclear receptor. The relative levels of expression of the different TR isoforms vary among cell types and stages of development, suggesting that they could have organ-specific functions. Thus, TRα1 is more highly expressed in bone, brain, intestine, hearth and skeletal muscle, whereas TRβ1 levels are higher in liver and kidney and the expression of TRβ2 is restricted to the anterior pituitary, and some neural cells (Lazar 2003; Pascual and Aranda 2013).

The possibility that TR isoforms could mediate specific actions is supported by gene inactivation experiments performed in mice. Studies with knockout (KO) mice for TRs obtained by homologous recombination have indicated that KO mice for α and β TR isoforms have different phenotypes. Deletion of TRα1 causes reduced body temperature and anomalous heart rate and contractility (Wikstrom *et al*. 1998), whereas TRβ KO mice gene present altered pituitary-thyroid axis and defects in the inner ear and retinal development (Forrest and Vennstrom 2000). Furthermore, in mice lacking both TRα1 and TRα2 postnatal development and survival is compromised (Fraichard *et al*. 1997), while the double TRα1/TRβ KO mice, surprisingly, survive indicating that these receptors are not essential for viability (Gothe *et al*. 1999). Furthermore, TRα1/TRβ KO mice show bone defects with abnormal growth and a severe dysfunction of the pituitary-thyroid axis, which are not present in the single KO mice. TRα1 and TRβ can play overlapping functional roles in organs such as the skin, since single KO mice present a proliferative defect but not as marked as that found in double KO mice (Contreras-Jurado *et al*. 2011; Garcia-Serrano *et al*. 2011). All these results indicate that TRα1 and TRβ can substitute for each other to mediate some actions of the thyroid hormones but they can also mediate isoform-specific functions.

Structure of the thyroid hormone receptors

TRs and other nuclear receptors exhibit a modular structure with different regions corresponding to autonomous functional domains that can be interchanged among related receptors without loss of function. A typical nuclear receptor consists of a variable N-terminal region (A/B), a conserved DNA-binding domain (DBD) or region C, a linker region D, and a conserved E region that contains the ligand binding domain (LBD) (Figure 2).

The A/B region is the most variable both in size and sequence and contains a ligand-independent transcriptional activation function (AF1). In contrast, the DBD is the most conserved domain among nuclear receptors, and confers the ability to recognize and bind with high affinity to the TREs. This domain comprises two "zinc fingers" and a carboxy terminal extension. In each zinc finger, 4 invariable cysteines coordinate tetrahedrically one zinc ion. Aminoacids required for discrimination of the TRE are present at the base of the first finger in a region termed the "P box", and other residues of the second zinc

finger that form the so called "D box" are involved in dimerization. Structural studies have shown that the core DBD is composed of two α helices. The first one, the recognition helix, that binds the major groove of DNA, contains the P box that makes contacts with specific DNA bases of the response element. The second helix contains the D box and forms a right angle with the recognition helix (Rastinejad *et al*. 1995).

The D domain is not well conserved among the different receptors and serves as a hinge between the DBD and the LBD, allowing rotation of the DBD. The D domain in TRs contains residues whose mutation abolishes interaction with nuclear receptor corepressors. In addition, this region associates strongly with the LBD but only in the presence of ligand or corepressors, exerting a stabilizing effect on the overall structure of the receptor (Pissios *et al*. 2000).

The LBD is a multifunctional domain that, besides the binding of the hormone, mediates homo and heterodimerization and ligand-dependent transcriptional activation and repression. The LBDs contains a carboxi-terminal AF2 motif responsible for ligand-dependent transcriptional activation. The crystal structure of the LBDs shows that are formed by twelve conserved α-helical regions numbered from H1 to H12. The ligand binding pocket, which accommodates the hormone, is mainly made up of non-polar aminoacids and is buried within the bottom half of the LBD. The AF2 domain, contained in H12, is required for ligand-dependent transactivation. This domain possesses a high homology over a very short region that adopts an amphipathic α-helical conformation. Although H12 of the LBD contains the core AF2 activity, this domain comprises other dispersed elements brought together upon hormone binding. One of such elements is a region encompassing the C-terminal half of helix 3 and helix 4. Mutations in this region affect neither ligand binding nor dimerization, but impair ligand-dependent transactivation. Specifically, a highly conserved lysine in the carboxi-terminus of helix 3 is important for transcriptional activity not only in TR but also in most receptors (Aranda and Pascual 2001).

Several differences are evident when comparing the unoccupied and ligand-bound receptors (Figure 3). The liganded structures are more compact than the unliganded ones, demonstrating that upon hormone binding the receptors undergo a clear conformational change. The most striking difference observed in the receptors upon ligand binding is the position of H12 (Wagner *et al*. 1995). This helix projects away from the body of the LBD in the absence of ligand. However, upon hormone binding H12 moves in a "mouse-trap" model being tightly packed against H3 or 4 and making direct contacts with the ligand. This change generates a hydrophobic cleft responsible for interaction with coactivators (Feng et al. 1998).

Dimerization and binding to TREs

In contrast with steroid receptors that almost exclusively recognize palindromic DNA elements, TRs can bind as homodimers or preferentially as heterodimers with other nuclear receptor, the retinoid X receptor or RXR, to TREs composed of two copies of the AGG/TTCA motif. They are configured as palindromes (Pal), inverted palindromes (IPs), or direct repeats separated preferably by 4 non-conserved base pairs (DR4) (Figure 4). Although TRs can bind to their response elements as homodimers, heterodimerization with RXR strongly increases the affinity for DNA and transcriptional activity. Therefore, RXR plays

a dual role in nuclear receptors signaling. On one hand, this receptor binds DNA as a homodimer and activates transcription in response to the ligand 9-*cis*-retinoic acid, and on the other hand serves as a heterodimeric partner for other nuclear receptors, including TRs. Since DRs are inherently asymmetric, heterodimeric complexes may bind to them with two distinct polarities. However, it has been established that on a DR4 RXR occupies the upstream half-site and the heterodimeric partner TR occupies the downstream motif (Kurokawa *et al*. 1993). The ability of the TR/RXR heterodimer to bind to palindromes, IPs and DR elements implies that the DBDs must be rotationally flexible with respect to the LBD dimerization interface and a different region of the DBD of each receptor must be used to create the dimerization interface (see Figure 4). Some receptor heterodimers can be indistinctly activated by ligands of either RXR or its partner receptor, and are synergistically activated in the presence of both ligands. In contrast, it was believed that formation of the TR/RXR heterodimer precluded binding of ligand to RXR, and that in these complexes RXR would to be a “silent partner” with the only function of increasing binding to the TRE. However, more recent data indicate that RXR can bind ligand and recruit coactivators as a heterodimer with TR, demonstrating that in particular cellular environments this receptor can act as a "non-silent" partner of TR, allowing stimulation by RXR agonists (Castillo *et al*. 2004).

Coactivators

The packing of DNA in nucleosomes provides a major obstacle for gene transcription. Two major mechanisms alleviate the block of transcription caused by the nucleosomal structure: histones can be post-translationally modified to destabilize chromatin, and nucleosomes can be disrupted through the activity of ATP-driven machines.

Histones are subjected to a great variety of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation. These modifications occur in a number of residues on the amino-terminal and carboxy-terminal histone “tail” domains, which play an essential role in controlling the folding of nucleosomal arrays into higher-order structures. Specific histone modifications can be associated to transcriptional activation, whereas others are associated with repression. This has led to the ‘histone code’ hypothesis (Jenuwein and Allis 2001), in which specific combinatorial sets of histone modification signals can dictate transcriptional activation or repression.

Chromatin of transcriptionally active genes has been known for a long time to be enriched in hyperacetylated histones. Histone acetylation is catalyzed by histone acetyltransferases (HAT), whereas acetyl groups are removed from the histone tails by the action of histone deacetylases (HADCs). The receptors do not possess HAT enzymatic activity that could modify the histones, but they associate with coactivator complexes with HAT activity. A well-known family of coactivators that are recruited to TRs and other nuclear receptors in a ligand-dependent manner is the SRC/p160 family with three related members: SRC-1/NCoA-1, SRC/2TIF-2/GRIP-1/NCoA-2 and p/CIP/ACTR/AIB1/TRAM1/RAC3. Studies performed in genetically modified mice show that despite their similarity p160 coactivators show non-redundant actions, since SRC-1 KO mice display resistance to TH (RTH) with increased circulating TSH levels despite an increase in serum free TH levels, whereas TIF-2 KO mice have normal thyroid

function (Weiss *et al*. 1999). In addition, deletion of both SRC-1 and TIF-2 results in marked increases of serum TH and TSH concentrations, and double heterozygous animals show a phenotype of resistance to TH similar to that found in SRC-1 KO mice. This indicates a gene dosage effect in nuclear coactivators manifesting as haploinsufficiency and functional redundancy of SRC-1 and TIF-2 (Weiss *et al*. 2002).

p160 coactivators possess HAT activity and act as primary coactivators, interacting with TRs and other nuclear receptors, but they also serve as platforms for the recruitment of secondary coactivators (York and O'Malley 2010). The p160 family of coactivators shows a conserved structure, with a nuclear receptor-interacting domain (RID) in their central region. The RID contains three highly conserved LxxLL motifs, where L is leucine and x is any aminoacid, and mediates association of the coactivators to hormone-bound receptors. The cocrystal structure of the LBD of TR and a p160 fragment containing two LxxLL motifs indicates that the conserved glutamic acid in H12 and lysine in H3 of the receptor make hydrogen bonds to leucines 1 and 5 of the coactivator RID. These contacts form a charge clamp that orients and positions the coactivator RID into the hydrophobic groove formed in the LBD after the conformational change elicited by ligand binding (Darimont *et al*. 1998). This explains why mutation of these residues in TRs renders a transcriptionally inactive receptor. Furthermore, mice homozygous for the knock-in mutation at E457 in H12 of TRβ, resulting in disruption with p160 coactivators, have high serum TSH and TH levels, consistent with resistance to TH (RTH) and demonstrating that the AF-2 domain is required for up-regulated and down-regulated TH action (Ortiga-Carvalho *et al*. 2005).

The N-terminal domain of p160 coactivators mediates interactions with several transcriptional activators, with BAF57 a component of ATP-dependent chromatin remodeling factors, and with a coiled coil coactivator (CoCoA). p160 coactivators also contain two domains with intrinsic transcriptional activity. The stronger transactivation domain (AD1) is the region of interaction with the HAT CBP/p300, which serves coactivator roles for many different types of transcription factors, acting as cointegrator of extracellular and intracellular signaling pathways and is also an essential coactivator for the receptors. p160 coactivators also associate with PCAF, the first identified mammalian HAT. Thus, p160 coactivators might serve as a docking platform to bridge protein complexes with HAT activity to the DNA-bound TR. Histone acetylation is a critical step in nuclear receptor-mediated hormone signaling and it has been shown that in vivo histone acetylation levels of nuclear receptor target genes is strongly induced upon treatment with the corresponding ligand.

Methylation of histones in arginine residues has been also demonstrated to be associated to gene activation (Lee and Stallcup 2009). Interestingly, a transactivation domain (AD2) located in the carboxi-terminus of p160 coactivators has been shown to interact with secondary coactivators with histone arginine methyltransferase (HMT) activity such as CARM1 (cofactor-associated arginine methyltransferase 1) and PRMT (protein arginine methyltransferase). Their coactivator potential is dependent on an intact histone methyltransferase domain, and CARM1, p160 and CBP/p300 act synergistically to enhance ligand-dependent transcriptional activation by the receptors (Lee *et al*. 2005).

Formation of the transcriptional initiation complex in RNA polymerase II dependent promoters requires binding of the general transcription factors (GTFs). Performed complexes, composed of the RNA polymerase II, GTFs, SRBs (supressor of RNA polymerase B) and several other proteins, termed “the holoenzyme”, can be directly recruited to the promoter by sequence-specific transcription factors. A multiprotein complex denominated TRAP (thyroid receptor associated proteins) that interacts with TR (and other nuclear receptors) in a ligand-dependent manner and enhances the ligand-dependent transcriptional activity has been isolated. This complex is equivalent to the yeast Mediator complex that together with SRB proteins associates with the large subunit of RNA polymerase. It is believed that the TRAP complex acts by recruiting the polymerase holoenzyme to the target promoter. The TRAP complex is recruited to the core AF2 TR region in response to hormone binding through a single subunit (TRAP220) via a LxxLL motif identical to that found in the p160 coactivators (Fondell *et al*. 1996; Yuan *et al*. 1998).

TH-dependent transcriptional activity requires recruitment of both TRAP and p160 coactivator complexes to TR. As both coactivator complexes interact with the same receptor region they could compete with each other for binding to the receptor. Studies employing chromatin immunoprecipitation (ChIP) assays have shown that p160 and TRAP coactivator complexes are recruited to hormone-regulated promoters in a sequential manner. p160 complexes act earlier than TRAP and this ordered recruitment may proceed for multiple cycles of factor association and dissociation, with multiple rounds of transcription occurring within each cycle (Metivier *et al*. 2006).

In summary, binding of hormone to TRs allows the recruitment of coactivators with unique biochemical activities at temporally appropriate times during the transcription process. The receptors can recruit first coactivators with HAT and arginine HMT activity resulting in histone acetylation and methylation. Once chromatin has been uncondensed, the receptors via their association with the TRAP complex would be able to recruit the transcriptional machinery resulting in stimulation of target gene expression (see Figure 5).

Corepressors

In addition to hormone-dependent gene activation, in the absence of ligand TRs can repress basal transcription of TRE-containing genes. Binding of hormonal ligand to the receptor releases the transcriptional silencing and leads to gene activation. The current model of gene regulation by these receptors assumes that the unliganded receptors are bound to the TRE and that under these conditions are associated with corepressors responsible for the silencing activity. The best characterized corepressors that associate with TR are 270-kDa cellular proteins named NCoR (nuclear corepressor) and SMRT (silencing mediator for retinoic and thyroid hormone receptors) (Chen and Evans 1995; Horlein *et al*. 1995). In contrast with coactivators, unliganded TRs interact strongly with corepressors and THs induce their dissociation from the receptors. NCoR and SMRT are related both structurally and functionally. They contain three autonomous repressor domains (RD) and a receptor interacting domain (RID) located toward the carboxyl terminus. The RID is composed of two motifs (or CoRNR boxes), with the consensus sequence Lxx I/H I xxx I/L. When compared to the LxxLL motif of the coactivators, the CoRNR motif presents an amino-terminal extended helix that appears to be required for effective binding

of the corepressors to the unliganded receptor (Perissi *et al*. 1999).

Although a receptor CoR box, located in H1 of the LBD within the hinge region, is essential for interaction of receptors with the corepressors, the CoRNR motif does not interacts directly with residues in this region, but docks to a hybrophobic groove in the surface of the LBD at H3 and 4. Since this surface overlaps with that involved in coactivator interaction, coactivator and corepressor binding is mutually exclusive. Indeed, the CoRNR motif binds the coactivator groove, preventing the AF2 H12 from assuming the active conformation.

Ligand binding by itself is not sufficient to induce dissociation of corepressors. Rather, it appears that the AF2 region serves to trigger the release of corepressors from the receptors. H12 is fully inhibitory for corepressor binding to most nuclear receptors, but deletion of this region allows corepressor interaction and in vivo repression. In the case of TRs, mutation or deletion of the AF2 domain increases interactions with corepressors and reduces the release of the corepressors after ligand binding, indicating again that H12 is inhibitory for corepressor recruitment (Hu and Lazar 2000; Sanchez-Martinez *et al*. 2008).

Transcriptional repression by the corepressor-bound receptors appears to be mediated by the recruitment of histone deacetylases (HDACs) to the target gene. Although corepressors were thought to act exclusively through the indirect recruitment of HDAC1 or 2 (class I deacetylases), via the adaptor mSin3 protein, the RD3 has been demonstrated to repress transcription by directly interacting with class II deacetylases (HDACs 4, 5 and 7). A repressor complex containing the corepressors, HDAC3 and transducin beta-like proteins (TBL1 or TBL1R) has been identified and appears to be required for repression by TR (Guenther *et al*. 2001; Yoon *et al*. 2003). TBL1 protein interacts with histone H3 and is associated with human sensorineural deafness. In vivo, TBL1 is bridged to HDAC3 through SMRT and can potentiate repression by the receptors. Furthermore, SNF2H, a component of chromatin remodeling complexes, is critical for TR mediated repression, showing that gene repression by the receptor involves the targeting of chromatin remodeling factors to the repressed gene by the HDAC activity of NCoR (Alenghat *et al*. 2006).

In summary, compactation of chromatin structure due to recruitment of HDAC complexes by the corepressors is involved in transcriptional silencing by the unliganded TRs. The conformational changes elicited in the receptors by TH binding would cause the dissociation of corepressors and the recruitment of coactivator complexes responsible for transcriptional activation (see Figure 5).

Although NCoR and SMRT are closely related, studies with KO mice suggest that they could mediate non-redundant biological actions. NCoR KO mice are embryonic lethal, suggesting the SMRT cannot compensate for the functions of NCoR in development and survival (Jepsen *et al*. 2000). On the other hand, knockin mice with mutations in the receptor interaction domain (RID) of SMRT and NCoR that abolish interaction with TRs show their importance in TR signaling (Astapova *et al*. 2008; Nofsinger *et al*. 2008). Liver specific expression of the mutant NCoR demonstrates that this corepressor plays an important in vivo role in transcriptional repression by the unliganded TR and that NCoR also mediates TH sensitivity on positive TR targets. When the mutant corepressor is ubiquitously expressed mice have low circulating TH levels, without TSH elevation, and markers of TH action in peripheral tissues do

not indicate hypothyroidism. These results indicate that NCoR mediates TH sensitivity in vivo and can alter the central set point of the thyroid axis. Interestingly, the mutant NCoR reversed much of the resistance phenotype seen in a *TR*β RTH mutant mouse model (Fozzatti *et al*. 2011), indicating that constitutive TR interaction with a corepressor is an important mechanism for RTH. Furthermore, the use a NCoR knockin mouse in which binding to HDAC3 is genetically disrupted shows that the interaction of NCoR with this deacetylase is also important for the control of both positively- and negatively-regulated genes by TH in vivo (You *et al*. 2010). In addition, NCoR appears to play a role in the oncogenic actions of a mutated TRβ in thyroid carcinogenesis in mouse (Furuya *et al*. 2007), and is much more potent than SMRT in the inhibitory actions of TRβ on cellular transformation by the *ras* oncogene (Garcia-Silva *et al*. 2011). Furthermore, NCoR but not SMRT has been implicated in thyroid hormone resistance (Yoh *et al*. 1997).

TH analogs

Although most residues in the ligand-binding pocket are conserved between TRα and TRβ, in the last 10-15 years it has been possible to develop isoform-specific ligands. TRβ selective agonists are particularly interesting because they have the potential to lower cholesterol and induce weight loss due to binding to hepatic TRβ, without having side effects in heart rate that is controlled by TRα (Baxter and Webb 2009; Webb 2010; Arsanjani *et al*. 2011). One of the best-characterized selective ligands is the TH analog GC-1 (Sobetirome), which shows approximately 10-fold higher affinity for TRβ than TRα (Scanlan 2010). Other thyromimetics such as GC24, or KB141 show an even higher selectivity. In some cases (KB2115, eprotirome), there is also a preferential concentration of the analog in the liver compared with the heart, and this also contributes to specificity. Other thyromimetic, MB07811, achieves liver specificity by being activated after entering hepatocytes by the action of cytochrome P450 to generate the TRβ agonist MB07344 (Erion *et al*. 2007). Thyromimetics have been employed in cultured cells, preclinical models and different clinical trials (Baxter and Webb 2009; Tancevski *et al*. 2011). Interestingly, in patients in which a statin was not very effective in lowering cholesterol, the combination with eprotirome produced a strong improvement of LDL levels (Ladenson *et al*. 2010), suggesting that TR agonists act by a mechanism different than that used by other cholesterol-lowering drugs. Very recently, it has been suggested that thyromimetics could be used as cholesterol-lowering drug in patients lacking LDL receptors for whom current therapies are ineffective. In KO mice lacking these receptors, GC-1 increases expression of cholesterol 7-α-monooxygenase, a protein involved in hepatic synthesis of bile acids from cholesterol and the amount of bile acids excreted in feces, lowering cholesterol (Lin *et al*. 2012). Although TRβ-specific agonists show high promise, further studies should establish the longer-term safety of these compounds in patients and their clinical value.

TRα and cardiac-selective ligands may have therapeutic utility in the area of heart disease**.** CO23 was the first thyromimetic developed showing TRα-specificity in vitro and in vivo and their derivatives CO24 and CO28 show enhanced TRα-specificity. Although their clinical importance has not ben tested, in tadpoles CO24 caused metamorphosis with precocious hind and fore leg emergence, consistent with enhanced TRα activation (Ocasio and Scanlan

2008).

TR-antagonists could hypothetically have clinical use in treating hyperthyroidism and the cardiac alterations associated with hyperthyroidism, such as ischemia and arrhythmias (Malm *et al*. 2009). TR antagonists such as NH3 have been designed and they induce a different receptor conformation than T3 (Figueira *et al*. 2011), but they are not specific for TRα or TRβ (Malm *et al*. 2009). However, and despite its significant potential usefulness, no TRα-selective antagonists are yet available and no TR-antagonists have been tested in clinical trials.

TR gene mutations

The syndrome of resistance to thyroid hormones (RTH), first described by Refetoff in 1967 (Refetoff *et al*. 1967), is characterized by a decreased sensitivity of tissues to the actions of thyroid hormone. The incidence of RTH is low (approximately 1 in 40,000), the syndrome is normally inherited in autosomal dominant manner, is present at similar frequency in males and females and is predominantly due to heterozygous mutations in *TRβ* (Refetoff and Dumitrescu 2007). However, in approximately 15% of the more than 1000 cases reported in the literature, mutations in the *TR* genes have not been found (Reutrakul *et al*. 2000), and the etiology is still unknown.

The first mutation identified was a single guanine-cytosine replacement in the codon for amino acid 340 resulting in a glycine-arginine substitution in the LBD of TRβ (Sakurai *et al*. 1989). We know now that in most families there are single nucleotide substitutions in *TRβ* causing point mutations, in some cases the syndrome is due to nucleotide deletions or insertions producing frameshifts, and only very rarely the mutation creates a stop codon. Many families share a common mutation and these mutations are concentrated in three clusters rich in CpG “hot spots”, present in the hinge domain and the LBD (Figure 6A). As can be deduced by the position of these mutations, the resulting receptors display a reduced hormone binding affinity or altered association with coactivators and corepressors. In many cases the mutant receptors show reduced interaction with coactivators or increased association with corepressors that are not released in the presence of hormone (Refetoff and Dumitrescu 2007).

Interestingly, whereas heterozygote patients with a mutant and a normal *TRβ* allele suffer RTH, total deletion of one allele does not cause the syndrome (Takeda *et al*. 1992). This is due to the fact that the presence of the mutant TRβ interferes with the action of the native receptor, a phenomenon known as “dominant negative effect” (Chatterjee *et al*. 1991). This explains why RTH due to *TRβ* mutation is inherited in a dominant form while inheritance is recessive in individuals with *TRβ* gene deletion. In addition, homozygous mutation of both alleles of the *TRβ* gene causes a very severe RTH (Ferrara *et al*. 2012) (Figure 6B).

Clinical diagnosis of RTH is based on the finding of high TH levels together with elevated TSH. This is explained because TRβ is responsible for TH feedback to TRH and TSH. In RTH the feedback is impaired because the mutant receptor cannot repress efficiently their expression and as a consequence of the inappropriate high levels of TSH the thyroid gland is hyperactive and more T4 and T3 are produced (Chiamolera and Wondisford 2009) (Figure 6C). In some patients the elevated levels of thyroid hormones can compensate for the defects in the receptor and they show a normal metabolic

activity with few clinical symptoms. The most common clinical signs in RTH patients include goiter and sinus tachycardia, and with less frequency they can have developmental delay, short stature, hearing loss, hyperactivity disorder, decreased IQ (but not overt mental retardation), and dyslexia (Refetoff and Dumitrescu 2007). However, by still unknown reasons, the clinical manifestations of the disorder are very variable even in subjects having the same *TRβ* mutation in the same family. In RTH patients coexist symptoms of hypo- and hyperthyroidism. The degree and type of affectation of the different tissues can be at least partially explained by the differential expression of TRβ and TRα. For instance, the heart is a TRα target organ and the elevated TH levels induce tachycardia as in hyperthyroid patients. As a consequence of TH binding to TRα in bone, a thyrotoxic skeletal phenotype can be also found in RTH. However, the high TSH levels, characteristic of hypothyroidism, reflect the dependence of the pituitary thyrotropes on TRβ.

Several knockin mice models mimicking human TRβ mutations have been generated to analyze the molecular mechanisms leading to RTH. The TRβPV mouse has a C-terminal 14-amino acid truncation deleting the AF2 domain (Kaneshige *et al*. 2000), and the TRβΔ337T mouse harbors a frame-shift mutation in the LBD that abolishes ligand binding and corepressor release (Hashimoto *et al*. 2001). Heterozygote mice manifest many of the abnormalities found in RTH patients, including elevated THs and TSH, neurological symptoms, abnormal growth or neurological dysfunction (Cheng *et al*. 2010). It has been shown that *in vivo* the mutant receptors exert their dominant-negative activity by competition with the native TRβ or TRα for binding to the TRE and for heterodimerization with RXRs (Hashimoto *et al*. 2001), leading to repression of the T3-positively regulated target genes. In addition, a mutant mouse with the R429Q mutation, which has normal ligand binding, but causes RTH in humans has been created. This mutation causes selective impairment of TH-mediated gene repression, while preserving gene stimulation, suggesting that the affected domain, necessary for TR homodimerization and corepressor binding, has a critical role in negative gene regulation by TH (Machado *et al*. 2009).

Until very recently no human mutations of the *TRα* gene were identified. This led to the hypothesis that either TRα mutations were lethal or that they could produce a phenotype different from that caused by TRβ mutation. Trying to clarify this point, different TRα knockin mice (TRα1PV, TRα1L400R and TRα1R384C), with mutations equivalent to others in TRβ causing RTH were generated (Kaneshige *et al*. 2001; Tinnikov *et al*. 2002; Liu *et al*. 2003). It was found that heterozygote mice were viable and that these mutations were associated with skeletal defects and other abnormalities different from those found in the TRβ mutants. Significantly, these animals do not present gross differences in the activity of the pituitary-thyroid axis, although T4 and T3 had a tendency to be low and high, respectively.

In 2012 two reports describe the first human patients with heterozygous TRα mutations (Bochukova *et al*. 2012; van Mullem *et al*. 2012). Both mutations E403X and F397fs406X map to the C-terminal domain of the receptor, and the single nucleotide change causes a premature stop in E403X and a frame-shift stop in F397fs306X (Figure 6D). The truncated receptors do not show ligand-dependent transactivation and have a strong dominant negative effect, inhibiting the activity of the native receptors. Since the mutation deletes H12 of TRα, the

resulting receptor does not recruit coactivators and displays enhanced corepressors recruitment that cannot be released in the presence of hormone.

In agreement with the findings in the TRα animal models, the pituitary-thyroid axis is not markedly dysregulated in the patients bearing heterozygous TRα mutations, explaining why no TRα mutations were identified before in RTH. However, TRα mutant patients present normal TSH levels together with subnormal reverse-T3, low-normal T4 and high-normal T3 levels, resulting in a low T4/T3 ratio. This has led to the suggestion that a reduced T4/T3 ratio together with subnormal reverse-T3 levels may represent a thyroid biochemical signature that facilitates future identification of additional cases (Schoenmakers *et al*. 2013).

The patients with TRα mutations present growth retardation, impaired bone ossification, constipation, bradychardia and low blood pressure, impaired motor coordination and mild cognitive deficits. These signs are suggestive of hypothyroidism, indicating severe resistance to THs in tissues such as bone, intestine, heart or brain expressing predominantly TRα. In contrast, tissues such as hypothalamus, pituitary or liver expressing predominantly TRβ do not show TH resistance, as demonstrated by a normal TSH suppression, reduction of cholesterol levels after T4 treatment or elevated levels of sex-hormone binding globulin (SHBG) (Bochukova *et al*. 2012; van Mullem *et al*. 2012).

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FIGURE LEGENDS

Figure 1. T4 and T3 enter the cell through transporter proteins. Inside the cells, deiodinases (DIO1,2) convert T4, the major form of thyroid hormone in the blood, to T3. DIO3 produces rT3 and T2 from T4 and T3, respectively. T3 binds in the nucleus to TRs that regulate transcription by associating, generally as heterodimers with RXR, to response elements (TREs) located in regulatory regions of target genes. Activity is regulated by an exchange of corepressor (CoR) and coactivator (CoA) complexes. Both receptors and coregulators are targets for phosphorylation by signal transduction pathways stimulated by extracellular signals. Binding of hormone to a subpopulation of receptors located outside the nuclei can also cause rapid "non-genomic" effects leading to stimulation of signaling pathways. T4 can also bind to integrin $\alpha V\beta 3$ inducing mitogen activated protein kinase (MAPK) activity.

Figure 2. Scheme of the main thyroid hormone receptors isoforms encoded by the *TR*α and *TR*β genes, depicting the functional domains. The A/B region contains the ligand-independent AF1 transactivation domain. The DNA-binding domain (DBD) or region C, is responsible for DNA binding. A linker region D connects the DBD to the conserved E/F region that contains the ligand binding domain (LBD) as well as the dimerization surface and the ligand-dependent AF2 transactivation domain. TRα1 and TRα2 vary in the C-terminal region and TRα2 cannot bind hormone. TRβ2 has a longer A/B domain and both isoforms can bind the thyroid hormone.

Figure 3. Schematic drawing of the receptor ligand-binding domain (LBD) in the absence and presence of hormone. α-helices are numbered from 1 to 12. Note the different position of the carboxy-terminal H12 that contains the core AF2 domain in the absence and presence of hormone that allows the exchange of corepressors (CoR) and coactivators (CoA)

Figure 4. TRs can bind as homodimers or as RXR heterodimers and can recognize diverse TREs in which the consensus AGGTCA motifs can be arranged as palindromes (Pal), inverted palindromes (IP), or direct repeats (DR) spaced by 4 non-conserved nucleotides.

Figure 5. In the absence of hormone the receptor heterodimer binds corepressors (SMRT/NCoR) that interact with histone deacetylases (HDACs) either directly or through their association with Sin3. Histone deacetylation causes chromatin compactation and transcriptional repression. Upon thyroid hormone binding, the receptors recruit different coactivator complexes. Some complexes contain histone acetyltransferases and arginine methyltransferases, others have ATP-dependent chromatin remodeling activity, and the TRAP/DRIP complex recruits the RNA polymerase II (RNAP II) holoenzyme to the target promoter. Recruitment of coactivators causes chromatin decompactation and transcriptional stimulation.

Figure 6. **A**) Scheme of TRβ showing the position of the three “hot spots” where mutations in the receptor are found in patients with RTH. **B**) The mutant receptors have dominant negative activity, causing RTH in heterozygote patients, no phenotype when only one normal allele is present, a very severe RTH when both alleles are absent. **C**) In RTH due to TRβ mutation, T4 and T3 levels are high due to a defective feedback inhibition on hypothalamus production of TRH and pituitary production of TSH. As a consequence TSH levels are high, despite the high levels of circulating thyroid hormones and enlargement of the thyroid gland can occur. **D**) Position of the mutations in TRα recently identified in patients. Mutations affect the C-terminus of the receptor containing the AF2 domain.

FIGURE 1

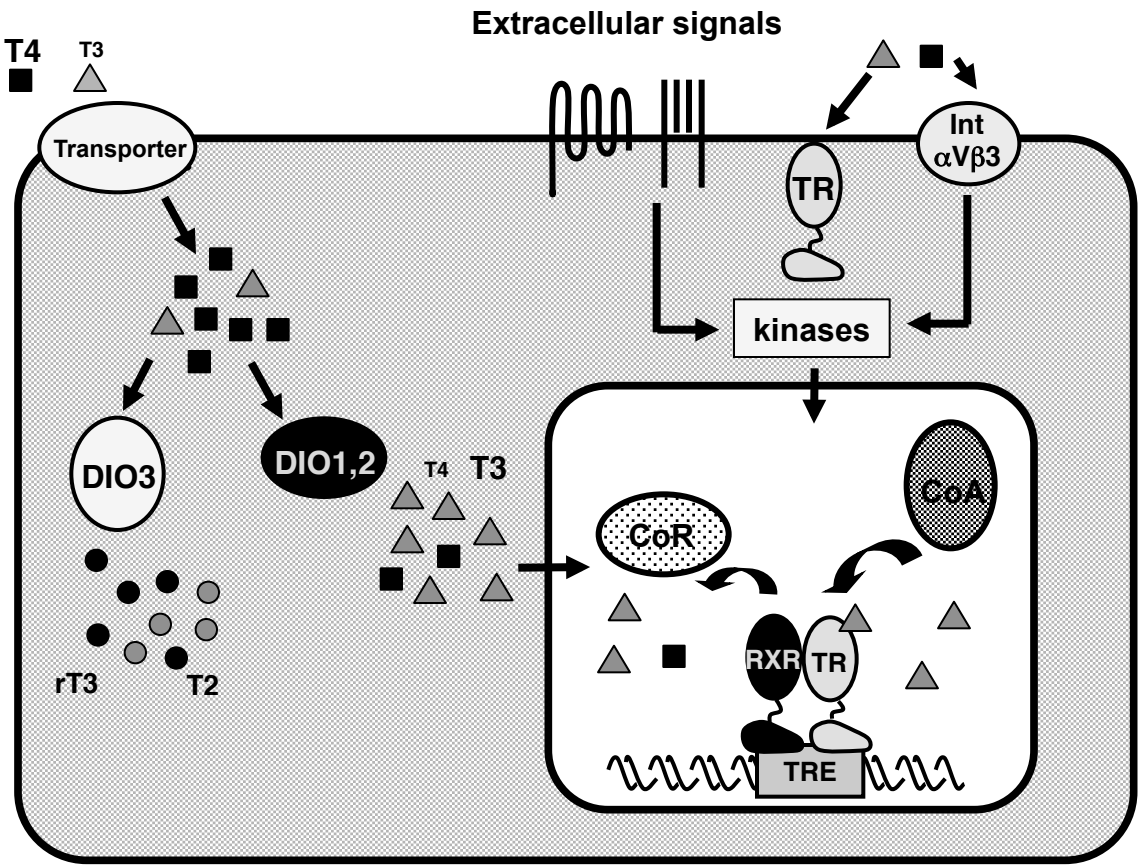
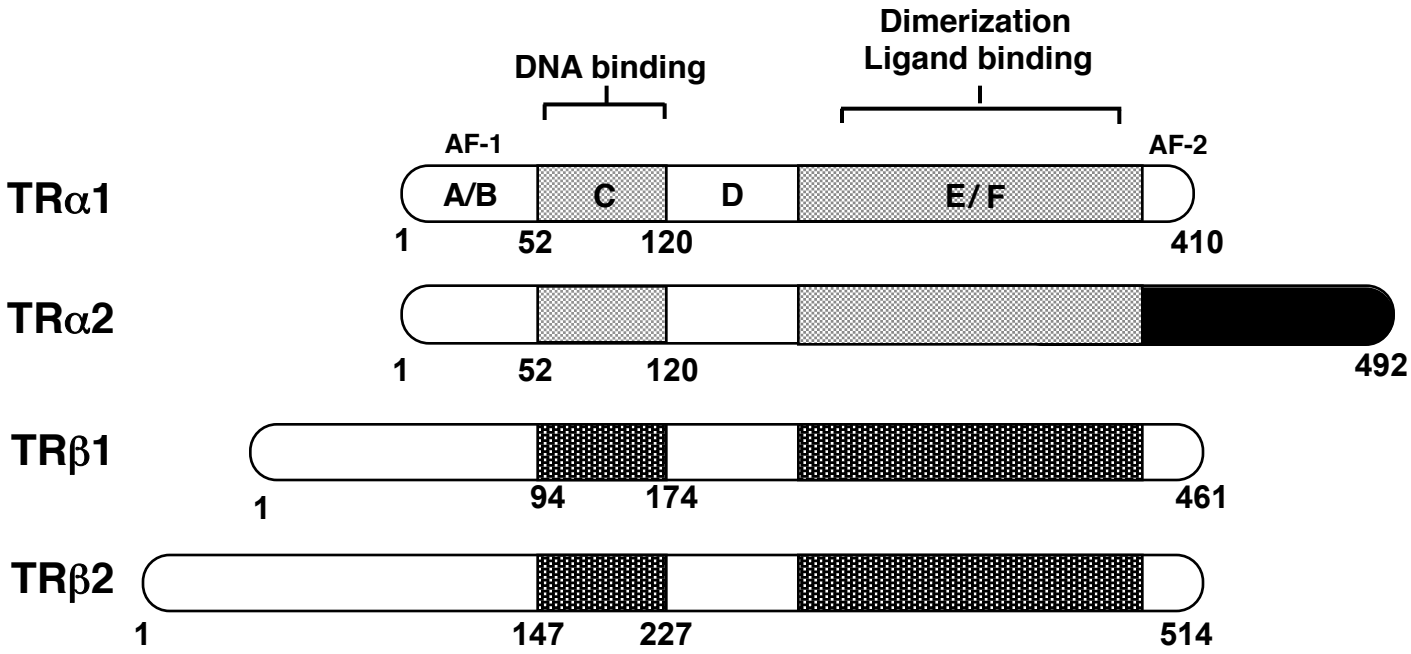

Extracellular signals
T4
T3
Transporter
Int
αVβ3
TR
kinases
DIO3
DIO1,2
T4
T3
CoA
CoR
RXR
TR
rT3
T2
TRE

FIGURE 2


Dimerization
Ligand binding
DNA binding
AF-1
AF-2
TRα1
A/B
C
D
E / F
1
52
120
410
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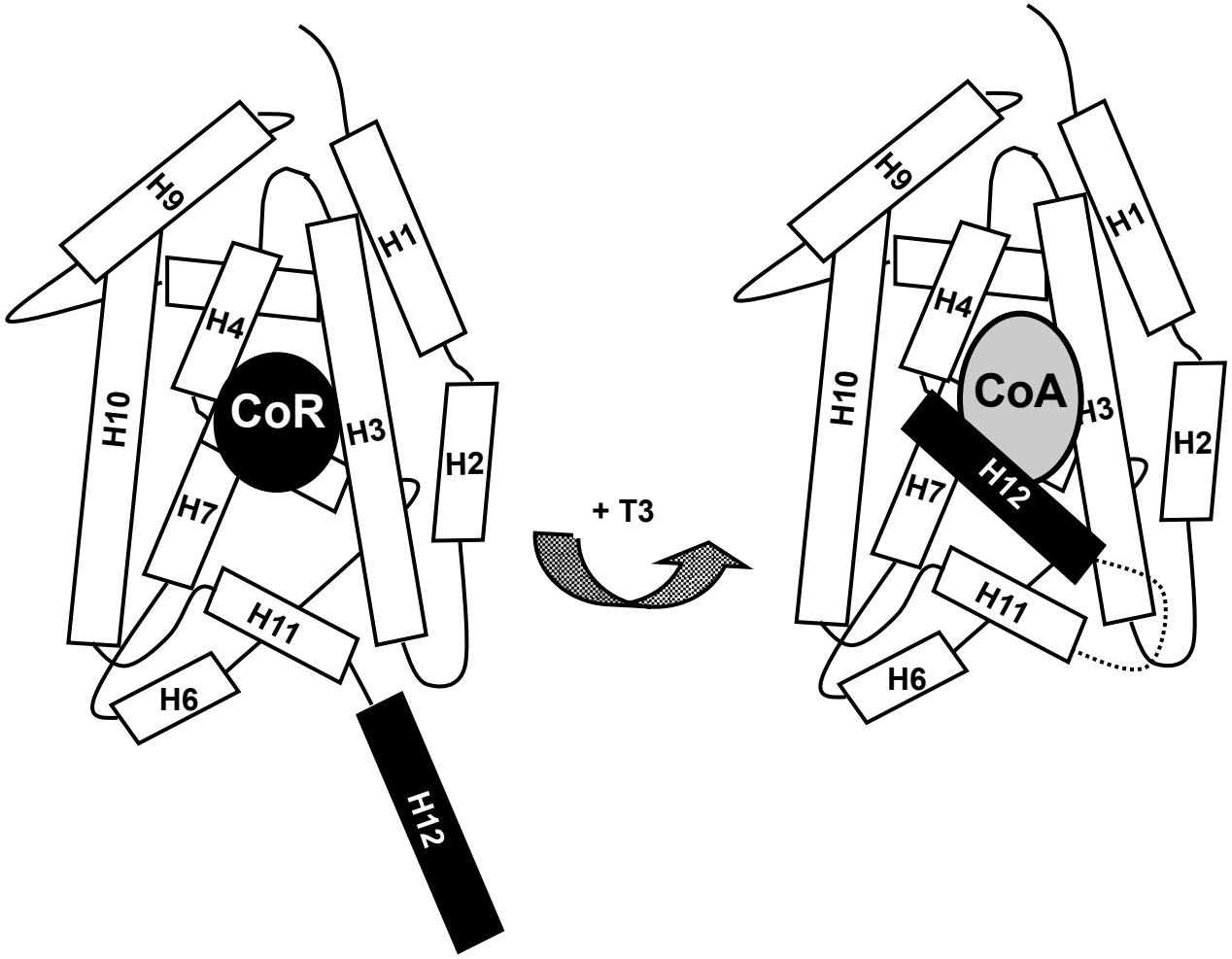
174
461
TRβ2
1
147
227
514

FIGURE 3


H9
H1
H4
CoR
H10
H3
H2
H7
H11
H6
H12
+ T3
H9
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H4
CoA
H10
H3
H2
H12
H7
H11
H6

FIGURE 4

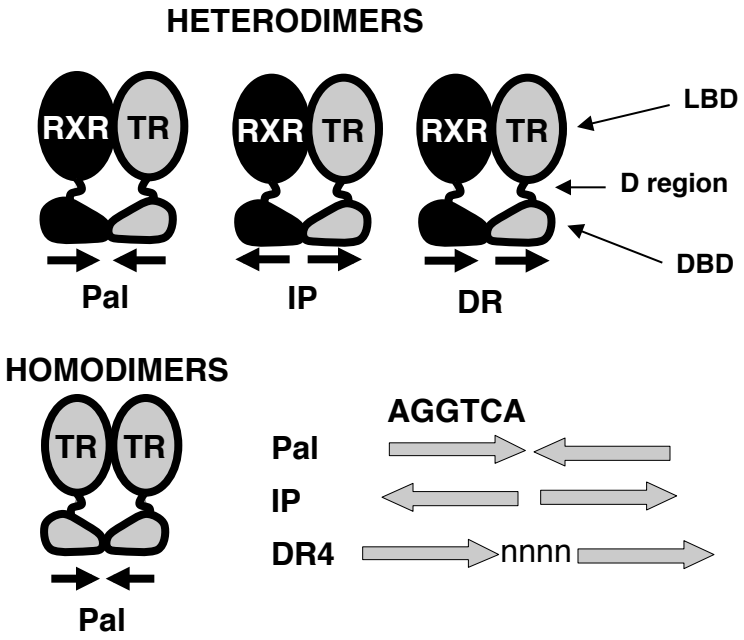

HETERODIMERS
RXR
TR
RXR
TR
RXR
TR
LBD
D region
DBD
Pal
IP
DR
HOMODIMERS
TR
TR
Pal
AGGTCA
Pal
IP
DR4
nnnn

FIGURE 5

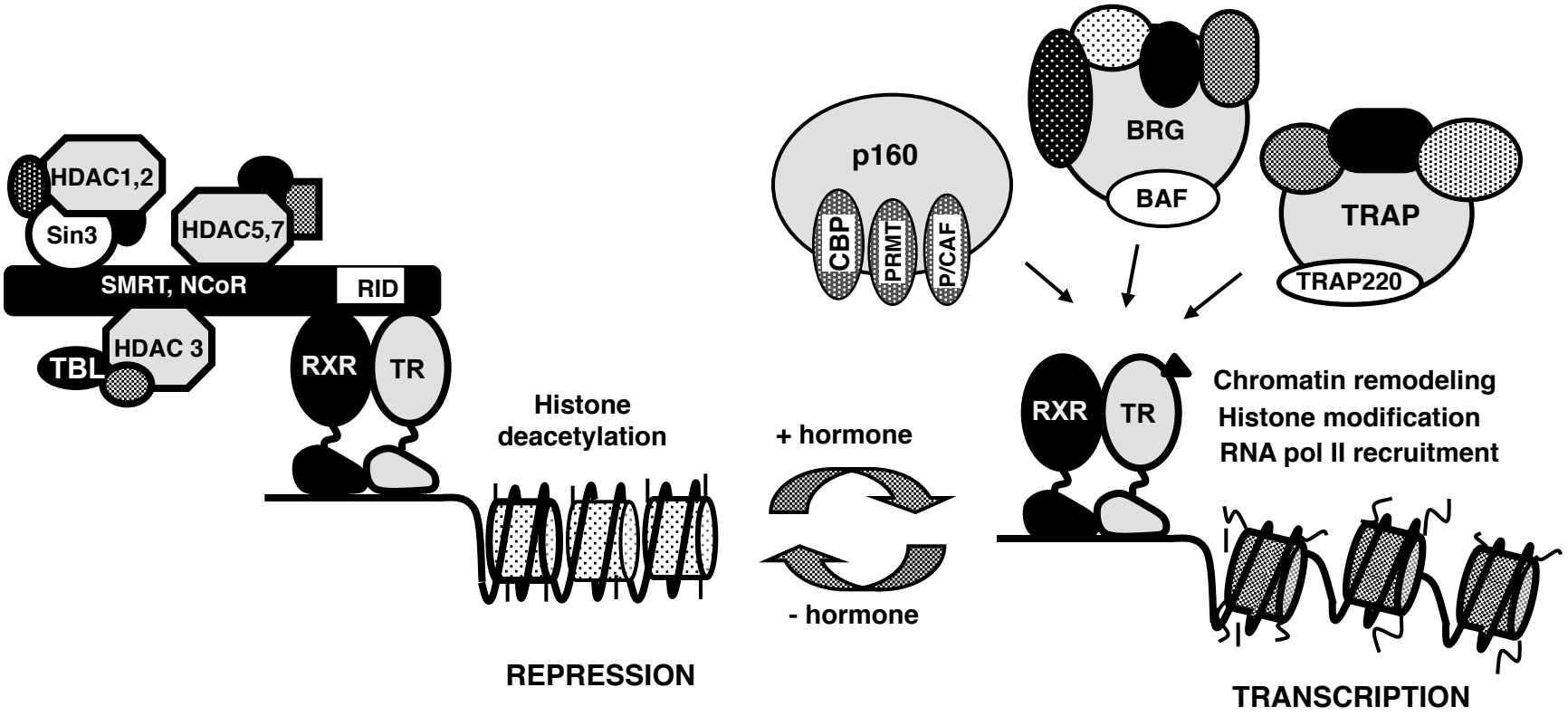
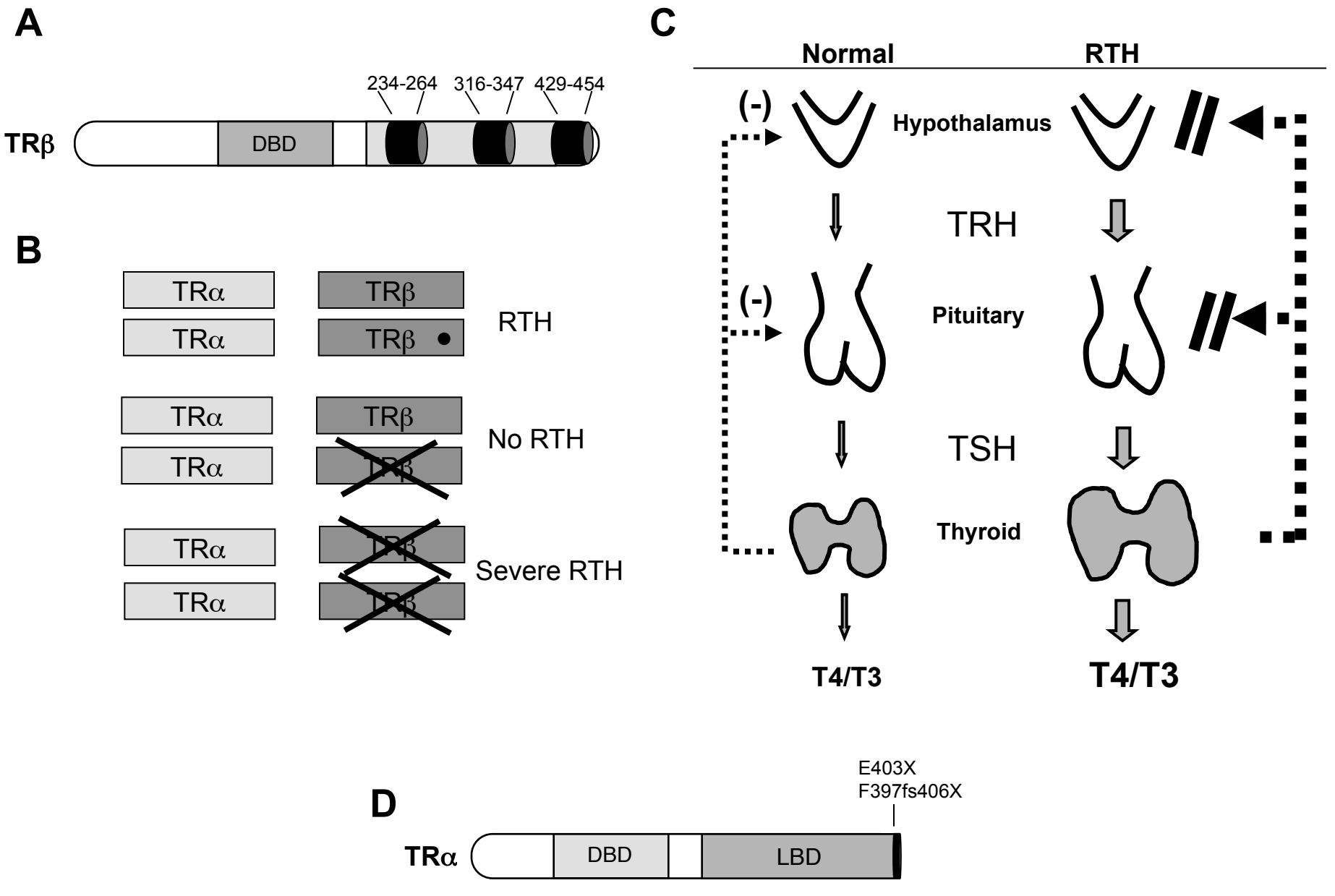

HDAC1,2
Sin3
HDAC5,7
SMRT, NCoR
RID
HDAC 3
TBL
RXR
TR
Histone
deacetylation
REPRESSION
+ hormone
- hormone
p160
CBP
PRMT
P/CAF
BRG
BAF
TRAP
TRAP220
RXR
TR
Chromatin remodeling
Histone modification
RNA pol II recruitment
TRANSCRIPTION

FIGURE 6


A
234-264
316-347
429-454
TRβ
DBD
B
TRα
TRβ
RTH
No RTH
Severe RTH
C
Normal
RTH
(-)
Hypothalamus
TRH
Pituitary
TSH
Thyroid
T4/T3
T4/T3
D
E403X
F397fs406X
TRα
DBD
LBD