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Thyroid hormone receptor localization in target tissues

Cyril S. Anyetei-Anum, **Vincent R. Roggero**, and **Lizabeth A. Allison**

Department of Biology, College of William and Mary, Williamsburg, Virginia, U.S.A

Abstract

The thyroid hormone receptors, TRα1, TRβ1, and other subtypes, are members of the nuclear receptor superfamily that mediate the action of thyroid hormone signaling in numerous tissues to regulate important physiological and developmental processes. Their most well-characterized role is as ligand-dependent transcription factors; TRs bind thyroid hormone response elements in the presence or absence of thyroid hormone to facilitate the expression of target genes. Although primarily residing in the nucleus, TRα1 and TRβ1 shuttle rapidly between the nucleus and cytoplasm. We have identified multiple nuclear localization signals and nuclear export signals within TRα1 and TRβ1 that interact with importins and exportins, respectively, to mediate translocation across the nuclear envelope. More recently, enigmatic cytoplasmic functions have been ascribed to other TR subtypes, expanding the diversity of the cellular response to thyroid hormone. By integrating data on localization signal motifs, this review provides an overview of the complex interplay between TR's dynamic transport pathways and thyroid hormone signaling activities. We examine the variation in TR subtype response to thyroid hormone signaling, and what is currently known about regulation of the variety of tissue-specific localization patterns, including targeting to the nucleus, the mitochondrion, and the inner surface of the plasma membrane.

Keywords

thyroid hormone; thyroid hormone receptor; nuclear import; nuclear export; mitochondrial import

Introduction

Thyroid hormone is essential for many diverse processes in nearly all vertebrate tissues, and abnormal thyroid hormone signaling underpins several human diseases (Chen, et al. 2013; Kim and Cheng 2013; Laudet and Gronemeyer 2002; Mendoza and Hollenberg 2017; Mondal, et al. 2016; Mullur, et al. 2014; van der Spek, et al. 2017). Much of thyroid hormone action is mediated by the thyroid hormone receptors (TRs), members of the nuclear receptor superfamily that act as ligand-dependent transcription factors. By modulating the transcription of target genes in response to ligand, TRs play key physiological roles in the regulation of many aspects of development, growth, and metabolism, including regulation of

Correspondence should be addressed to: L. A. Allison, Department of Biology, College of William and Mary, P.O. Box 8795, Williamsburg, VA, 23185, U.S.A., laalli@wm.edu.

Declaration of interest

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mitochondrial activity (Bernal 2017; Flamant and Gauthier 2013; Pascual and Aranda 2013; Skah, et al. 2017; Vella and Hollenberg 2017; Wrutniak-Cabello, et al. 2017). Thyroid hormone signaling is typically classified into two distinct pathways, nongenomic and genomic; however, these designations do not fully capture the subtleties of thyroid hormone action. To address the complexity of thyroid hormone signaling, a more precise nomenclature has recently been formulated (Flamant, et al. 2017). In this new classification scheme, four types of thyroid hormone signaling are defined: type 1 is the canonical pathway in which liganded TR binds directly to DNA; type 2 describes liganded TR tethered to chromatin-associated proteins, but not bound to DNA directly; type 3 suggests that liganded TR can exert its function without recruitment to chromatin in either the nucleus or cytoplasm; and type 4 proposes that thyroid hormone acts at the plasma membrane or in the cytoplasm without binding TR, a mechanism of action that is emerging as a key component of thyroid hormone signaling (Davis, et al. 2016; Kalyanaraman, et al. 2014).

The biological effect of thyroid hormone in a given tissue depends on a number of factors: the amount of available hormone, the levels of different TR subtypes and their post-translational modifications, the type of heterodimerization partner, and their interaction with corepressors and coactivators (Morte and Bernal 2014). In addition, accurate translocation of TRs from their synthesis in the cytosol to their ultimate destination is essential for maintaining proper cellular functions and activities (Bonamy and Allison 2006; Bonamy, et al. 2005; Bondzi, et al. 2011; Fernandez-Majada, et al. 2007; Wang and Li 2014). The thyroid hormone receptors are remarkably dynamic proteins. Although primarily residing in the nucleus TRα1 and TRβ1 shuttle rapidly between the nucleus and cytoplasm, and recent characterization of TRα1 isoforms with cytoplasmic functions adds a surprising twist to the intricacies of the receptor's subcellular trafficking. The fine balance between nuclear import and export of TRs has emerged as a critical control point for modulating thyroid hormone-responsive gene expression (Roggero, et al. 2016; Subramanian, et al. 2015), while an additional layer of complexity is added by multiple modular, often overlapping, functional domains. General understanding of nuclear localization signal (NLS) and nuclear export signal (NES) structure, mitochondrial and membrane targeting signals, and how these motifs are regulated will assist in refining understanding of the mechanism of action of TRs. In this review we will focus on mechanisms regulating the journey of TR from its site of synthesis in the cytoplasm to its final localization in target tissues, and how the receptor integrates gene expression across multiple levels in the cellular response to hormone. Before considering the cellular response to thyroid hormone, it is important to first examine the pathway by which thyroid hormone reaches target tissues and gains access to its intracellular receptors.

Thyroid hormone signaling

Thyroid hormone is produced through a feedback loop that includes the hypothalamus, pituitary, and thyroid gland, commonly referred to as the hypothalamic-pituitary-thyroid (HPT) axis (Medici, et al. 2015; Mendoza and Hollenberg 2017). The HPT axis involves a series of signal transduction cascades, where a signal sent from the hypothalamus eventually arrives at the thyroid gland, triggering release of thyroid hormone. In the circulatory system, the majority of total 3,5,3′,5′-L-tetraiodothyronine (thyroxine, T_4) and 3,5,3′-L-

triiodothyronine (T_3) are bound with three different thyroid hormone carrying proteins: thyroxine-binding globulin, transthyretin, and human serum albumin (Mondal et al. 2016; Pappa, et al. 2015). Upon reaching the target tissue, thyroid hormones enter cells via uptake through specific membrane transporters, including the monocarboxylate transporters MCT8 and MCT10 (Abe, et al. 2012; Bernal, et al. 2015). The most extensively characterized transporter, MCT8, transports thyroid hormone exclusively and preferentially binds T_3; however, secondary thyroid hormone transporters have been described that can compensate for loss of MCT8 expression, including the heterodimeric L-type amino acid transporters (LATs), LAT1 and LAT2, and the organic anion-transporting polypeptide (OATP) family (Mendoza and Hollenberg 2017).

Once in the cell, the intracellular concentration of thyroid hormone can be modified by the action of a suite of deiodinases. The prohormone T_4 can be converted to the physiologically active hormone T_3, or inactivated via conversion to 3,3′,5′-L-triiodothyronine (reverse T_3, or rT_3) within the cell. T_3 and rT_3 can be modified to form the physiologically active 3,5′-L-diiodothyronine (T_2), or the inactive 3,3′-L-diiodothyronine (3,3′-T_2), respectively, to protect tissues from excess hormone (Dentice, et al. 2013; Mondal et al. 2016; Orozco, et al. 2014). Whether T_4 is directly involved in mediating gene expression remains a subject of debate. T_4 is thought to primarily influence gene expression indirectly by cross-talk with other cell signaling pathways at the plasma membrane (Davis et al. 2016); however, there also is accumulating evidence that T_4 can directly modulate gene expression, dependent on the TR subtype and other cellular cofactors (Galton 2017). T_3 is directly involved in mediating gene expression by binding to TR in either the cytoplasm or nucleus of the cell (Bunn, et al. 2001). The intricate balance between thyroid hormone production and deiodination is critical for regulation of TR-mediated gene expression, and the dysregulation of this process may contribute to type II diabetes mellitus, obesity, cardiovascular disease, and some types of cancer (Brent 2012; Kim and Cheng 2013; Ruiz-Llorente, et al. 2011).

In addition to the type 1 canonical response mediated by nuclear TRs, thyroid hormone also has effects not exerted through the nuclear TRs; such effects were puzzled over early on to explain observations that thyroid hormone can, in some cases, initiate cellular responses that are too rapid to be attributed to transcription and translation (Davis et al. 2016; Flamant 2016). Although detailed coverage of type 4 actions of thyroid hormone is beyond the scope of this review, it is worth noting the existence of a hormone receptor that is associated with the plasma membrane structural protein αvβ3 integrin, a regulator of cell-cell and cell-extracellular matrix interactions (Cvoro, et al. 2016; Davis et al. 2016; Lin, et al. 2016; Martin, et al. 2014; Mullur et al. 2014). This receptor binds T_3 and T_4 and stimulates certain cellular responses, such as the remodeling of the actin cytoskeleton that is a vital component of brain development in neurons and glial cells (Leonard and Farwell 1997), and changes in the morphology of breast cancer cells (Flamini, et al. 2017). The αvβ3 integrin-associated receptor has two thyroid hormone binding sites, S1 and S2, that lead to activation of the phosphatidylinositol 3-OH kinase (PI3K) and ERK1/2 signaling pathways, respectively. The receptor is structurally unrelated and has no sequence homology to nuclear TR and, although it could be referred to as a “thyroid hormone receptor,” this nomenclature should be avoided to prevent misconceptions about the nature of this noncanonical receptor. Type 3 signaling,

mediated by transcriptionally inactive cytoplasmic TR isoforms, will be addressed later in this review.

Nuclear localization and function of thyroid hormone receptors

The type 1 genomic effects of TRs are two-fold; TRs can act as repressors of specific genes in the absence of ligand and activators of these same genes in the presence of ligand. For some genes, the reverse is the case: unliganded TR acts as an activator, while liganded TR is a repressor. This dual role of TRs implies constitutive nuclear localization. Many studies early on in the field supported this restricted subcellular distribution for TR (Andersson and Vennstrom 1997; Kumara-Siri, et al. 1986; Lee and Mahdavi 1993; Macchia, et al. 1992; Zhang and Lazar 2000; Zhu, et al. 1998). However, we and others have shown that even though TRα1 and TRβ1 appear to be predominantly nuclear at steady state, in fact, the receptors are undergoing rapid nucleocytoplasmic shuttling in both the presence and absence of T_3 (Baumann, et al. 2001; Bunn et al. 2001), movement which can be visualized by heterokaryon assays or fluorescence recovery after photobleaching (Grespin, et al. 2008; Subramanian et al. 2015). Detailed investigation of TRs has revealed distinct, dynamic localization patterns for some variants. Analysis of the intracellular localization of TRs by biochemical fractionation, immunocytochemistry, or indirect immunofluorescence assays has proved challenging overall, because of a lack of validated isoform-specific antibodies, and the difficulty in detecting endogenous TR subtypes that are less abundant in cells. Many studies have thus relied on transient transfection assays and expression of fluorescent protein-tagged TRs. With regards to nuclear localization, in our hands, there is no indication that overexpressing TRs leads to a more cytoplasmic localization by saturating the capacity of cells to transport proteins into the nucleus, or that fluorescent protein tags alter localization. For example, in transfected NIH-3T3 (mouse) cells or HeLa (human) cells, neither of which express detectable levels of endogenous TR, both exogenous GFP-tagged TRα1 and untagged TRα1 detected by antibody staining show a primarily nuclear distribution at steady state (Bonamy et al. 2005; Bunn et al. 2001).

Thyroid hormone receptor subtypes

The thyroid hormone receptors are well conserved throughout vertebrate evolution, originating from a single TR gene early in animal evolution (Manzon, et al. 2014); and there is evidence for nuclear TR-mediated responses to thyroid hormone in non-vertebrate lineages, including molluscs, echinoderms, cephalochordates, and ascidians (Darras, et al. 2011; Huang, et al. 2015; Laudet and Gronemeyer 2002; Taylor and Heyland 2017). The vertebrate thyroid hormone receptors are encoded by two genes located on different chromosomes, *NR1A1* and *NR1A2*, although due to ancestral gene duplication, some nonmammalian vertebrate species, including teleost fish, have two TRα-encoding genes (Darras et al. 2011; Galay-Burgos, et al. 2008). From these loci, a surprisingly diverse set of TR proteins are produced, through alternative splicing, alternative promoter usage, and internal initiation codons. Intense investigation of rodent and human TRs continues to reveal new subtypes, while the number of subtypes identified in other species, as of yet, is more restricted (Buchholz, et al. 2006; Kanaho, et al. 2006; Nelson and Habibi 2008; Politis, et al. 2017). For example, chickens and ducks have at least three subtypes (TRα, TRβ2, TRβ0)

(Bishop, et al. 2000); zebrafish produces two TRβ variants and at least three TRα isoforms that all act as functional nuclear receptors (Darras et al. 2011); two distinct TRα transcripts and one TRβ transcript have been isolated from the American alligator (Helbing, et al. 2006); and the Atlantic halibut has two TRα and two TRβ isoforms (Galay-Burgos et al. 2008). The main focus of this review is on the well-characterized mammalian receptors, in particular TRα1 and TRβ1.

Not all of the mammalian TR proteins produced act as nuclear receptors, however, and the physiological significance of many of the nonreceptor isoforms remains a subject of investigation (Flamant and Gauthier 2013; Mullur et al. 2014; Vella and Hollenberg 2017). What is currently known about the intracellular localization and function of the mammalian TRs is summarized in Table 1, and further described herein. The predominant isoforms generated by alternative splicing mechanisms include the *bona fide* nuclear receptors TRα1, TRβ1, TRβ2, TRβ3, and TRβ4 (Moriyama, et al. 2016; Tagami, et al. 2010); and the nonreceptor TR variants that lack T_3 binding ability, TRα2, TRα3, and TRα-ΔE6 (Casas, et al. 2006).

TRα1 has the highest expression in bone, the gastrointestinal tract, cardiac and skeletal muscle, and the central nervous system; TRα2 and TRα3 are predominant in the brain, kidney, testis, brown adipose tissue, and skeletal muscle (Guissouma, et al. 2014; Skah et al. 2017). TRα-ΔE6 is expressed in all tissues tested, and can sequester TRα1 in the cytoplasm (Casas et al. 2006). TRα2 is found consistently in mammals but not in other species. Although a dominant negative function has been attributed to mammalian TRα2, which is widely co-expressed with TRα1, the physiological relevance has remained a puzzle, particularly since it is unexplained why it would be necessary for TRα2 to counter-balance normal TR activity in mammals but not in non-mammalian species (Vennstrom et al., 2010). There is recent compelling evidence, however, that TRα2 modulates thyrotropin releasing hormone gene expression in the hypothalamus (Guissouma et al. 2014). In addition, four truncated forms of TRα1 (full-length, 46 kD) originate from alternative internal AUG translation initiation codons in TRα1 mRNA and are named based on their molecular masses: p43 starts at the equivalent of methionine-39 (Met^{39}) in the full-length receptor, p33 starts with Met^{120}, p30 starts with Met^{122}, and p28 starts with Met^{150} (Kalyanaraman et al. 2014; Wrutniak-Cabello et al. 2017) (Fig. 1). Finally, other truncated forms of TRα, TRΔα1 and TRΔα2, are produced from an internal promoter in intron 7; they contain only the C-terminus of the LBD and are expressed in the brain, lung, and gut (Davis et al. 2016; Chassande et al. 1997). TRΔα1 has been proposed as a candidate mediator of T_4-binding in the cytoplasm, potentially playing a role in regulating actin polymerization (Davis et al. 2016).

TRβ1 is most abundant in the liver, kidney, and the inner ear; TRβ2 is predominant in the hypothalamus, pituitary, cochlea, and retina; and TRβ4 is ubiquitously expressed, with relatively high expression in the brain and kidney (Flamant and Gauthier 2013; Hahm and Privalsky 2013; Mullur et al. 2014; Vella and Hollenberg 2017). Other minor isoforms of TRβ1 (52-kD) also exist; for example, two isoforms are alternatively translated from TRβ1 mRNA, with TRβ3 (44.6-kD) appearing to act as a functional receptor in rat (Flamant and Gauthier 2013), and TRΔβ3 (32.8-kD) functioning as a ligand-responsive dominant negative

antagonist (Williams 2000). In addition, an elongated form of TRβ2, termed TRβ2Δ, has been proposed to function as a nuclear receptor in the rat pituitary gland (Zhao, et al. 2014). In this review, we will focus on the TRα and TRβ isoforms where intracellular localization and targeting signals have been investigated in more detail.

Functional domains of the thyroid hormone receptor

The thyroid hormone receptor consists of four modular domains that are evolutionarily conserved among the nuclear receptor superfamily (Fig. 1): a variable N-terminal A/B domain, which contains a region involved in transactivation, activation function-1 (AF-1); a central DNA binding domain (DBD) comprised of two zinc fingers; a C-terminal ligand-binding domain (LBD), which also includes dimerization interfaces and activation function-2 (AF-2); and a linker or hinge region between the LBD and DBD that contributes to DNA binding, activation function and repression, ligand binding, and corepressor interactions (Mondal et al. 2016; Nascimento, et al. 2006; Pawlak, et al. 2012; Zhang et al. 2017). TRα1 and TRβ1 both contain AF-1 domains involved in the transcriptional response to hormone; while the TRβ2 isoform, which differs from TRβ1 in the A/B domain, has a unique hormone-independent AF-1 domain that recruits coactivators (Tomura, et al. 1995; Oberste-Berghas et al. 2000).

Ligand-binding domain conformation

The LBD of TR is composed of 12 α-helices that form a hollow pocket lined with hydrophobic residues. The ligand binding site is highly flexible, and the structural details underpinning receptor activation after T_3 binding are complex (Schweizer, et al. 2017). The twelfth helix contains the ligand-dependent activation domain, AF2 (Figueira, et al. 2011). Helix 12 forms a short pivoting structure that can adopt different conformations. In the absence of T_3, helix 12 is in an extended position and the corepressor binding groove is occupied by the corepressor nuclear receptor (CoRNR)-box helical motifs found in silencing mediator for retinoid or thyroid-hormone receptors (SMRT) and nuclear receptor co-repressor 1 (N-CoR1). Binding of T_3 may induce a hormone-dependent “mouse-trap” mechanism (Flamant 2016; Moras and Gronemeyer 1998; Sonoda, et al. 2008), where helix 12 rotates to swing shut and close off the pocket around T_3. As a result of this conformational change, a novel docking surface forms for interaction with LXXLL motifs (L denotes leucine; X denotes an undetermined amino acid) of a transcriptional coactivator (Rosen and Privalsky 2011). A refinement of this model suggests that TR helix 12 functions as a “selective gatekeeper” that actively discriminates between different forms of corepressor even in the unliganded receptor (Rosen and Privalsky 2009); and other models propose that rearrangements in a mobile part of the LBD comprising helix 3, the loop between helix 1 and helix 2, and nearby β-sheets, play a greater role in ligand dissociation than repositioning of helix 12 (Martinez, et al. 2006). Mutations that disrupt helix 12 alter corepressor specificity as well as T_3-mediated release of corepressors (Rosen and Privalsky 2009). Recent X-ray crystallographic structural studies have revealed a second ligand binding site in TR located between helices 9–11 that may interact with T_4 (Souza, et al. 2014).

Nuclear import and export signals

The nuclear transport process provides a central regulatory point for coordinating cell signaling and gene expression. Macromolecules known as nuclear pore complexes (NPCs) are the regulatory gatekeepers of the entry and exit of nuclear proteins, and allow for the passive diffusion of small molecules less than 40-kD (Li, et al. 2016). NPCs are distributed throughout the nuclear envelope, embedded at sites within the luminal space between the outer and inner membrane of the nuclear envelope (Cautain, et al. 2015; Tran, et al. 2014). They are octagonally symmetric cylindrical structures made up of proteins termed nucleoporins or Nups, that act to anchor the NPC in the nuclear envelope and provide interaction domains for nuclear proteins to translocate through a central channel (Hayama, et al. 2017). The translocation of nuclear proteins through the NPCs is typically facilitated by karyopherin β-like family members (importins and exportins), with each member performing a distinct nuclear import, export, or bidirectional transport function (Chook and Suel 2011; Kimura and Imamoto 2014).

Our systematic characterization of nuclear export signal (NES) and nuclear localization signal (NLS) motifs by site-directed mutagenesis has elucidated the mechanics of TR nuclear localization (Mavinakere, et al. 2012). In depth analysis of TRα1 and TRβ1 structure reveals that the two subtypes both contain a classical bipartite NLS, named NLS-1, residing in the hinge region, and a second monopartite NLS, termed NLS-2, located in the A/B domain of TRα1 that is absent in TRβ1 (Fig. 1) (Mavinakere et al. 2012). RNAi and coimmunoprecipitation assays show that members of the importin family of karyopherins, specifically importin 7, importin β1, and adapter importin α1 recognize these NLSs and directly mediate the nuclear import of TRs through the NPC (Roggero et al. 2016) (Fig. 2). In support of the importance of NLS-1 for efficient nuclear localization, an isoform that lacks the hinge domain, TRα-ΔE6, has a strikingly altered localization compared with TRα1; TRα-ΔE6-GFP was shown to be predominantly expressed in the cytoplasm with minor nuclear fluorescence (Casas et al. 2006). In addition, TRβ4 is primarily localized to the nucleus, and mutation of two putative NLSs near the hinge region results in a whole cell distribution of the receptor (Moriyama et al. 2016).

In an earlier study, we showed that TRα1 exits the nucleus through two pathways, one dependent on the export factors CRM1 and calreticulin, and the other CRM1-independent (Grespin et al. 2008). In a subsequent study, we also identified a novel NES in helix 12 of the ligand-binding domain of TR (NES-H12). Another novel NES motif spans helix 3 and helix 6 (NES-H3/H6) (Mavinakere et al. 2012) (Fig. 1). Notably, these NES motifs are not sensitive to leptomycin B, a specific inhibitor of CRM1, suggesting that they mediate the CRM1-independent export pathway followed by TR. Follow-up work by RNAi has shown that multiple exportins influence TR export, including exportins 4, 5, and 7 (Subramanian et al. 2015). Not surprisingly, the two NLSs found in TRα1 act to confer strong nuclear localization to the receptor; we hypothesize that TRβ1's small cytosolic population (Baumann et al. 2001; Zhu et al. 1998) may reflect an altered balance of NLS and NES activity (Mavinakere et al. 2012; Zhang et al. 2017) (Fig. 3). Although multiple NLS and NES motifs exist in a variety of nuclear proteins, how these multiple signals interact in collective remains unclear (Bonaldi, et al. 2003; Dai, et al. 2015; Lu, et al. 2014; Mavinakere

et al. 2012; Panayiotou, et al. 2016; Umemoto and Fujiki 2012). Once TRs are directed into the nucleus and released from importin, they can then interact with target genes to modulate gene expression in response to hormone.

Thyroid hormone receptor gene activation and silencing

A multifaceted cascade of events results in binding of TRs to thyroid hormone response elements (TREs) and culminates in the modulation of target gene expression in response to thyroid hormone (Ayers, et al. 2014; Bernal and Morte 2013; Brent 2012; Vella and Hollenberg 2017). Thyroid hormone receptors often heterodimerize with the retinoid X receptor (RXR), expanding the range of T_3 responsiveness for genes within the same cell (Diallo, et al. 2007; Flamant 2016). On positive TREs, corepressors, such as N-CoR1 or N-CoR2 (also known as SMRT) and histone deacetylase (HDAC), are bound in the absence of ligand to TR, leading to repression of target gene expression (Mendoza, et al. 2017; Oberoi, et al. 2011; Xu, et al. 1999). Upon ligand binding, TR undergoes a conformational change, resulting in a new set of activator proteins bound to the receptor, such as SRC-1 (p160/steroid receptor coactivator 1) and histone acetyltransferase (HAT). This leads to changes in chromatin structure and the subsequent transcription of the target gene (Dasgupta and O'Malley 2014; McKenna, et al. 1999; Soriano, et al. 2011). In addition to unliganded TR bound to positive TREs, chromatin immunoprecipitation sequencing (ChIP-seq) analysis of endogenous TR in mouse liver tissue suggests that the receptor's interaction with chromatin is highly dynamic and that it can be recruited to chromatin in a ligand-dependent manner (Grontved, et al. 2015). These findings align with an earlier report that used fluorescence recovery after photobleaching (FRAP) to show that TRβ1 moves rapidly within the nucleus, and that ligand binding does not affect its mobility (Maruvada, et al. 2003). A recent study in mice suggests that TR target genes respond to T_3 based on the availability of specific corepressors and coactivators, providing an explanation for tissue-specific responses to similar amounts of T_3 (Vella, et al. 2014). In addition to activating transcription on positive TREs, TRs can also repress gene expression, possibly by binding to putative negative TREs in a T_3-dependent manner (Bernal and Morte 2013). In this instance, N-CoR1 and SMRT appear to play a role in determining T_3-sensitivity, suggesting that corepressors can be recruited to TR in the presence of T_3 (Astapova and Hollenberg 2013; Astapova, et al. 2011; Shimizu, et al. 2015). The mechanism remains unclear, however, and a recent genome-wide analysis of chromatin occupancy of TRs in neural cells does not appear to support the hypothesis that liganded TR acts directly as a transcription repressor (Chatonnet, et al. 2013). Further, ChIP-seq studies in hypothyroid and hyperthyroid mouse liver cells suggest that negative regulation instead may be mediated by diminished TR recruitment in the presence of T_3 (Ramadoss, et al. 2014).

Cytoplasmic Functions of the Thyroid Hormone Receptor

For many years the focus in the field was on characterizing the nuclear function of TRs, but now their emerging roles in the cytoplasm also must be considered. Study of the functional domains of full-length TRα1 (p46) and the truncated isoforms p43, p33, p30, and p28 has revealed conflicting intracellular targeting signals within TRα1 that can direct the proteins to the nucleus, mitochondria, or the inner surface of the plasma membrane (Kalyanaraman et

al. 2014; Mavinakere et al. 2012; Wrutniak-Cabello et al. 2017) (Figs. 1 and 4). TRα1 p43 and p28 are targeted to the mitochondrial matrix and mitochondrial inner membrane, respectively. The biological function of TRα1 p33 remains unknown, but p30 is post-translationally modified via palmitoylation and colocalizes with caveolin-1 at the inner surface of the plasma membrane. Upon binding T_3 the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signaling cascade is activated, and stimulates proliferation and survival in multiple cell types (Hiroi, et al. 2006; Kalyanaraman et al. 2014; Wrutniak-Cabello et al. 2017).

Studies in diverse cells types, including human adipose-derived stem cells (hADSC), human primary osteoblasts, mouse osteoblast-like MC3T3 cells, monkey kidney cells (CV-1), neonatal rat ventricular myocytes (NRVM), and mouse cardiomyocytes (HL-1), have revealed TR subtypes localized to the mitochondria, plasma membrane, and cytoplasmic compartments in a tissue-specific manner (Carazo, et al. 2012; Cvoro et al. 2016; Kalyanaraman et al. 2014; Wadosky, et al. 2016). Of particular interest, human ADSCs are multipotent adult stem cells with the capacity to differentiate into adipocytes, chondrocytes, and osteocytes, and they express TRα1, TRα2, and TRβ1 at variable levels. TR intracellular localization was investigated by indirect immunofluorescence assay and, interestingly, all subtypes showed cytoplasmic localization. Further examination via double immunostaining of TRα1 and TRα2 with a mitochondrial marker showed a predominantly mitochondrial localization for TRα1 proteins (Carazo et al. 2012; Psarra and Sekeris 2008; Wadosky et al. 2016). Although western blot analysis was not performed to visualize protein size, these findings suggest that truncated forms of TR were reliably being detected by the antibodies used in this study.

Mitochondrial targeting

A major compartment of thyroid hormone accumulation within the cell is the mitochondria (Bassett, et al. 2003; Davis et al. 2016; Psarra and Sekeris 2008; Wrutniak-Cabello et al. 2017). The major effect of thyroid hormone on mitochondrial activity has been partially explained by reports of truncated TRα1 variants localizing to the mitochondria of different mammalian tissues, such as liver, brown and white adipose tissue, red and white muscle, heart, tongue, and testis (Carazo et al. 2012; Fumel, et al. 2013; Wrutniak-Cabello et al. 2017). In addition, a truncated TRβ ($TR_{\beta}A1$) localizes to the mitochondria in *Xenopus laevis* (South African clawed frog) oocytes (Saelim, et al. 2007). TRα1 p43 is targeted to the mitochondrial matrix, while TRα1 p28 is targeted to the mitochondrial inner membrane (Carazo et al. 2012; Kalyanaraman et al. 2014; Wrutniak-Cabello et al. 2017) (Fig. 4). TRα1 p43 displays an N-terminal deletion that lacks NLS-2, but still possesses NLS-1 in the hinge region (Fig. 1). In contrast, TRα1 p28 displays an N-terminal deletion of the A/B domain, the DBD, and NLS-1. Neither p43 or p28 possess a canonical mitochondrial import signal (MIS). Nonetheless, sequences within helices 5, 10, and 11 in the C-terminal LBD of p43 and p28 have been identified that are necessary for mitochondrial import (Carazo et al. 2012) (Fig. 1). Helix 5, spanning amino acids 242–252 of TRα1, was found to drive an atypical mitochondrial import process independent of ATP and the mitochondrial membrane potential; whereas helices 10–11, spanning amino acids 298–354, induced a typical mitochondrial import process sensitive to ATP and the mitochondrial membrane potential.

Whether these two mitochondrial import sequences, MIS1 and MIS2, are functional or not, is proposed to depend on the “permissive” role of the N-terminus of TRα1 (Carazo et al. 2012). In this model, conformational changes of the protein, dependent on the flexibility of the hinge region, would disrupt the functionality of NLS-1 in the hinge region and induce the activity of the mitochondrial import sequences (Wrutniak-Cabello et al. 2017). Interestingly, TRβ1 harbors these conserved MIS1 and MIS2 motifs and lacks NLS-2 in the N-terminal A/B domain (Fig. 1), but there is no evidence of functionality of the MIS motifs. It is of interest to determine the exact nature of the N-terminal A/B domain sequence in regulating localization of TRs to the mitochondrial or nuclear compartments.

Plasma membrane targeting

Beyond type 1 genomic actions within the mitochondria, type 3 actions of TR are primarily associated with its localization to the plasma membrane. The alternative translation product TRα1 p30 is targeted to the plasma membrane where it is proposed to play a key role in mediating signaling pathways involved in cell survival and proliferation (Carazo et al. 2012; Kalyanaraman et al. 2014; Wrutniak-Cabello et al. 2017). Further, there is tissue-specific variation in p30’s localization to the plasma membrane (Kalyanaraman et al. 2014). In murine primary osteoblasts, TRα1 p30 associates with lipid rafts (cholesterol-rich plasma membrane microdomains that contain caveolin-1) to function as a unique signal transduction platform. In contrast, in MC3T3 cells TRα1 p30 associated with caveolin-1, nitric oxide synthase 3 (NOS3), protein kinase G type II (PKGII), and the tyrosine kinase Src. These data point to the possibility that TRα1 p28 localizes to the mitochondrial inner membrane following a similar mechanism (Kalyanaraman et al. 2014), and provide an understanding of how certain membrane-targeted proteins interact with caveolin to reach the plasma membrane (Hayer, et al. 2010).

A role for posttranslational modification in TR localization

Post-translational modifications (PTMs) play a significant role in the regulation of protein structure, enzymatic activity, stability or degradation, subcellular localization, protein-protein interactions, and diverse cell signaling (Azevedo and Saiardi 2016; Drazic, et al. 2016; Lin, et al. 2015; Rodriguez 2014). Many amino acid side chains such as cysteine (C), serine (S), threonine (T), and tyrosine (Y) are post-translationally modified; however, the amino acid lysine (K) is targeted by an extremely high number of PTMs including methylation, ubiquitination, sumoylation, and acetylation. Thyroid hormone receptors, and other nuclear receptors, undergo PTMs that influence transcriptional activity and subcellular localization (Abdel-Hafiz and Horwitz 2014; Cui, et al. 2004; Faresse 2014; Lin, et al. 2005; Sanchez-Pacheco, et al. 2009). For example, the association of TRα1 p30 with the plasma membrane is mediated by palmitoylation, a post-translational lipid modification. Consequently, it has been predicted that cysteine (Cys)^{254} and Cys^{255} palmitoylation is necessary to localize p30 to the plasma membrane (Kalyanaraman et al. 2014).

For nuclear TRs, phosphorylation regulates DNA binding and transcriptional activation, and it has been shown that phosphorylation of one or more sites in TRα1 enhances nuclear retention or inhibits nuclear export but is not directly involved in nuclear import (Nicoll, et

al. 2003). Intriguingly, a recent study suggests the phosphorylation of TRβ1 may play a role in promoting nuclear localization in serum-starved Chinese hamster ovary (CHO) cells. FLAG-tagged TRβ1 was shown to form a cytoplasmic complex with the p85 regulatory subunit of PI3K and the Src family kinase Lyn (Martin et al. 2014). Complex formation was dependent on two phosphotyrosine motifs in the second zinc finger of TRβ1 that are not conserved in TRα1. When hormone was added, the complex dissociated, allowing PI3K activity to increase and TRβ1 to move into the nucleus to regulate transcription. It will be of interest to extend these studies to tracking receptor movement in live cells. The authors suggest that dramatic shifts in localization may not be observable with GFP-tagged receptors, because the GFP tag might interfere with PI3K association; however, their qualitative observations of receptor distribution are consistent with the variability we see in populations of cells expressing GFP-TRβ1. As shown in Fig. 3, GFP-TRβ1 typically has a greater cytosolic population than GFP-TRα1, and we find TRβ1 distributions ranging from whole cell to primarily nuclear. For critical analysis of the fine nuances of receptor localization, rigorous quantification of the nucleus versus cytoplasmic distribution by fluorescence intensity measurements will be essential.

Acetylation sites that are important for transcriptional activity have been identified in the hinge domain of TR, corresponding to K130, K134, and K136 in human TRα1 (Sanchez-Pacheco et al. 2009), and to K184, K188, and K190 in TRβ1 (Lin et al. 2005). These lysines are integral components of NLS-1 (Mavinakere et al. 2012), suggesting that acetylation state could have an impact on NLS activity. Whether this PTM is important for modulating the nuclear localization of TR subtypes is under investigation.

It is known that ubiquitination of liganded TRα1 targets the receptor for rapid proteasome-mediated degradation (Bondzi et al. 2011). Recently, it was reported that monoubiquitination of TRα1 within its LBD results in a shift in the diffuse intranuclear localization of TRα1 toward the nuclear periphery in cardiomyocytes (Wadosky et al. 2016). TRα1 activity stimulates hypertrophy in cardiomyocytes, and although TRα2 and TRβ1 are present in this cell type, they lack this function. Muscle-specific ubiquitin ligase muscle ring finger-1 (MuRF1) (Rodriguez, et al. 2015) was shown to monoubiquitinate TRα1 *in vitro*; however, specific lysine sites have not yet been identified and monoubiquitinated forms have not been detected *in vivo* (Wadosky et al. 2016). Whether polyubiquitination or monoubiquitination directly modulates TR nucleocytoplasmic shuttling remains to be determined.

Several studies have provided evidence that sumoylation of TR plays an essential role in fine-tuning TR regulation of gene expression. SUMO modification sites have been identified at K283 and K389 of TRα1 (positioned in NES-H3/H6); and at K50 (A/B domain within AF1), K146 (DBD) and at K443 (near the NES-H12 motif) of TRβ1 (Liu, et al. 2015; Liu, et al. 2012; Weitzel 2016). Given the proximity of the SUMO-modified lysines to NES motifs, sumoylation is also under study for its impact on NES activity and TR nuclear localization.

Taken together, these reports provide insights into the possible interplay of TR post-translational modification with TR localization: palmitoylation directs p30 to the membrane; phosphorylation promotes nuclear retention; acetylation occurs within the hinge NLS-1; and ubiquitination and sumoylation occur within the NES-containing LBD of TR. Although not

yet reported to be post-translationally modified, TRα1 p43 contains mitochondrial import sequences and thus has a high probability of also containing PTM sites that modulate trafficking.

Mislocalization of thyroid hormone receptors and disease

In addition to diseases correlated with dysregulated hormone production, mutations in TR can give rise to disease, most notably the autosomal dominant Resistance to Thyroid Hormone (RTH) syndrome; and mutations can contribute to certain types of cancer, including human hepatocellular carcinoma, renal clear cell carcinoma, breast cancer, pituitary tumor, and thyroid cancer (Astapova et al. 2011). Early evidence to suggest that mutated TR could be involved in carcinogenesis came from the discovery that TRα1 is the cellular counterpart of the retroviral v-ErbA carried by the avian erythroblastosis virus involved in acute erythroleukemia and sarcomas (Sap, et al. 1986). Many of these TR mutants have lost T_3 binding and transactivation capacity and some exhibit dominant negative activity (Chan and Privalsky 2010; Conde, et al. 2006; Kim and Cheng 2013; Lin, et al. 2013; Martinez-Iglesias, et al. 2009; Rosen, et al. 2011; Rosen and Privalsky 2009, 2011; Wojcicka, et al. 2014). The question is thus raised, does receptor localization impact disease pathology? So far, the answer appears to be, yes. Dominant negative TR mutants, such as v-ErbA, have been shown to localize to both the nuclear and cytoplasmic compartments in cells (Boucher, et al. 1988), are recruited to aggresomes, display altered transport activity and mislocalize TRα1 to these cytosolic inclusions (Bonamy and Allison 2006; Bonamy et al. 2005; Bondzi et al. 2011; Bunn et al. 2001; DeLong, et al. 2004; Takalo, et al. 2013; Zhang et al. 2017). The altered localization of v-ErbA appears to be enhanced by acquisition of the N-terminal viral Gag sequence, which harbors a strong CRM1-dependent NES (DeLong et al. 2004).

The factors that determine whether a given amino acid substitution causes endocrine disruption or cancer remain enigmatic, particularly for changes within the LBD. Typically, human cancers have multiple TR mutations, while single mutations are characteristic of RTH, and it has been proposed that synergistic interactions of these mutations strengthen the dominant negative activity (Rosen and Privalsky 2009, 2011). RTH syndromes exist due to mutations in the respective TR isoforms, TRα1 and TRβ1, and the variability in symptomatic phenotype is characterized by the tissues in which these isoforms are highly expressed (Mendoza and Hollenberg 2017; Mullur et al. 2014; Vella and Hollenberg 2017; Vella et al. 2014). Clinical phenotypes of RTH include elevated thyroid hormone levels, goiter, short stature, decreased weight, tachycardia, hearing loss, attention-deficit hyperactivity disorder, decreased IQ, and dyslexia (Bochukova, et al. 2012; Dumitrescu and Refetoff 2013; Moran, et al. 2013; Parrilla, et al. 1991; Schoenmakers, et al. 2013). Interestingly, the highest frequency of mutations occurs in the region corresponding to NES-H12, with another cluster of mutations occurring within NES-H3/H6 (Fig. 1).

Except for our prior studies, there is little information on the contribution of altered nucleocytoplasmic shuttling dynamics to the phenotype of RTH and cancer-promoting mutants. Two of our recent findings stand out: a R26H substitution in NLS-2 of the oncoprotein v-ErbA abrogates the activity of NLS-2, while mutagenesis studies on NES-

H12 point to the intriguing possibility that altered shuttling of TRβ1 may be a contributing factor in RTH (Mavinakere et al. 2012). Based on these data, we hypothesize that intracellular mislocalization of TR is a crucial factor to consider in pathogenesis (Bonamy and Allison 2006; Bonamy et al. 2005).

Concluding remarks

Thyroid hormone receptor subtypes mediate the actions of thyroid hormone in a variety of cellular compartments, including the nucleus, the mitochondria, and at the inner surface of the plasma membrane (Fig. 4). Within the nucleus, TRα1 and TRβ1 bind to the TREs of target genes, in the presence or absence of thyroid hormone, to influence an astonishing number of cellular processes, including cell proliferation, oxygen consumption, protein synthesis, and carbohydrate, lipid, and vitamin metabolism. The physiological significance of TRα1 and TRβ1 nucleocytoplasmic shuttling may, at least in part, be to serve as a "ferry boat" (Kolodkin, et al. 2010) to increase the rate of T_3 (and possibly T_4) nuclear entry, relative to simple diffusion through the cytosol, or to circumvent localization of T_3 to the mitochondria. Furthermore, important PTMs have been reported that suggest an increasingly complex interplay with TR's NLS and NES motifs, and possibly MIS motifs, that may affect TR's ultimate localization in target tissues. There is a dynamic balance between nuclear import, retention, and export of shuttling transcription factors and, in the case of TR, localization to cytosolic compartments as well. These observations, coupled with the multiplicity of thyroid hormone signaling within the cell, may provide important insights into the development of treatments for RTH and some types of cancer.

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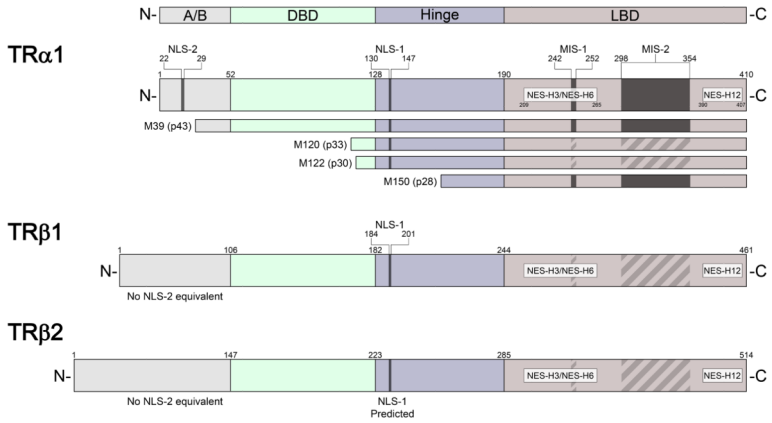

Figure 1.

Thyroid hormone receptor (TR) major subtypes and localization signals. The structural diagram (not to scale) of TRα1, TRβ1, and TRβ2 shows nuclear localization signal (NLS), nuclear export signal (NES), and mitochondrial import sequence (MIS) motifs, where known (solid bar) or predicted (striped bar) based on sequence homology. The positions of localization signals are indicated in relation to the respective individual domains of TR: N-terminal A/B domain (A/B), DNA-binding domain (DBD), hinge domain, and ligand-binding domain (LBD). The TRα1 mRNA encodes several forms of truncated TR by translation initiation from internal AUG sites encoding methionine (M); amino acid residue numbers correspond to the position in full-length TRα1.

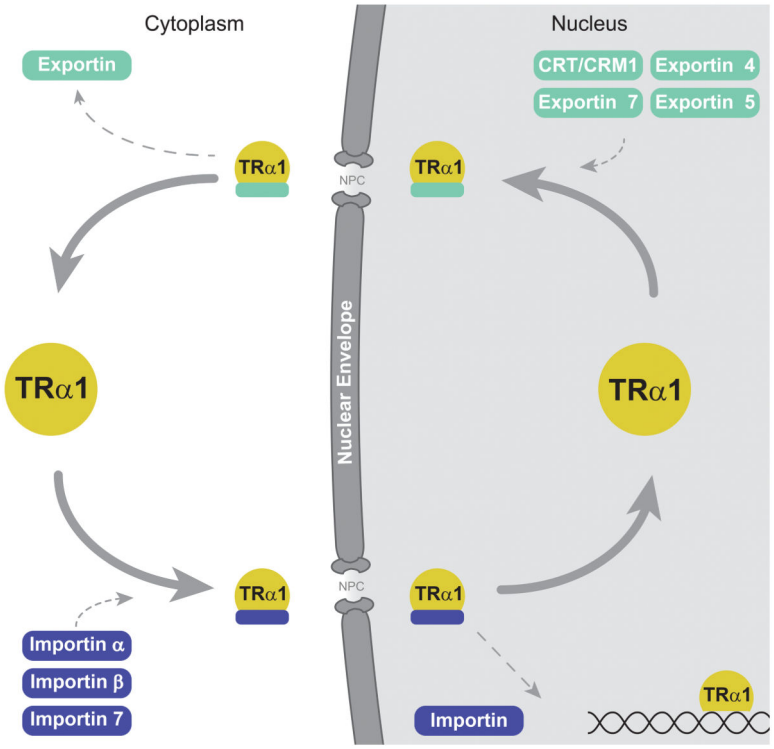

Figure 2.

Thyroid hormone receptor nucleocytoplasmic shuttling pathway. The well-characterized pathway for TRα1 is depicted. TRα1 binds to specific importins in the cytoplasm, as indicated. The TRα1-importin complex passes through a nuclear pore complex (NPC) embedded in the nuclear envelope into the nucleus, where the complex is disassembled and TRα1 binds to target genes. TRα1 exits the nucleus through the NPC in association with specific exportins or calreticulin (CRT)/CRM1. TRβ1 follows a similar nucleocytoplasmic shuttling pathway, but nuclear import is solely mediated by the importin α1/importin β1 complex.

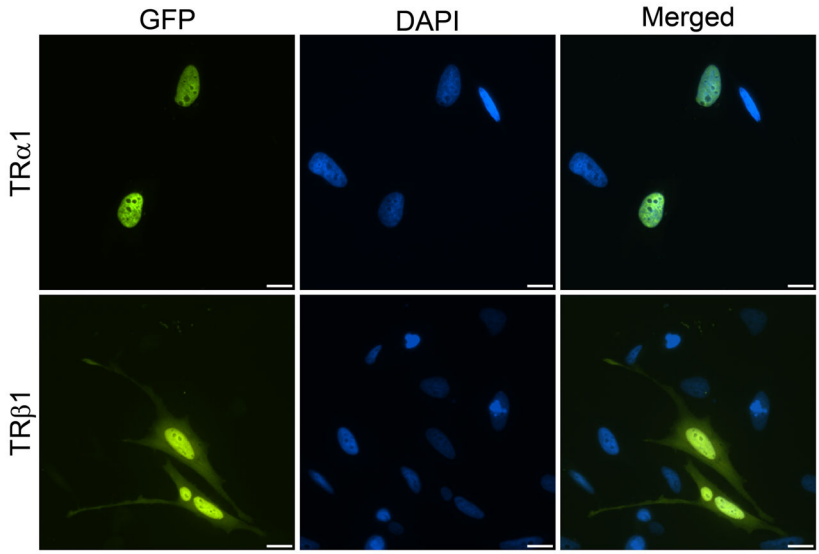

Figure 3.

Distinct intracellular localization patterns for TRα1 and TRβ1. HeLa cells transfected with expression plasmids for green fluorescent protein (GFP)-tagged TRα1 and TRβ1 were analyzed by fluorescence microscopy after staining for DNA with DAPI to visualize the nucleus. GFP-TRα1 predominantly localizes to the nucleus; GFP-TRβ1 also localizes to the nucleus but has a slight cytoplasmic population. Scale bar, 10 μm.

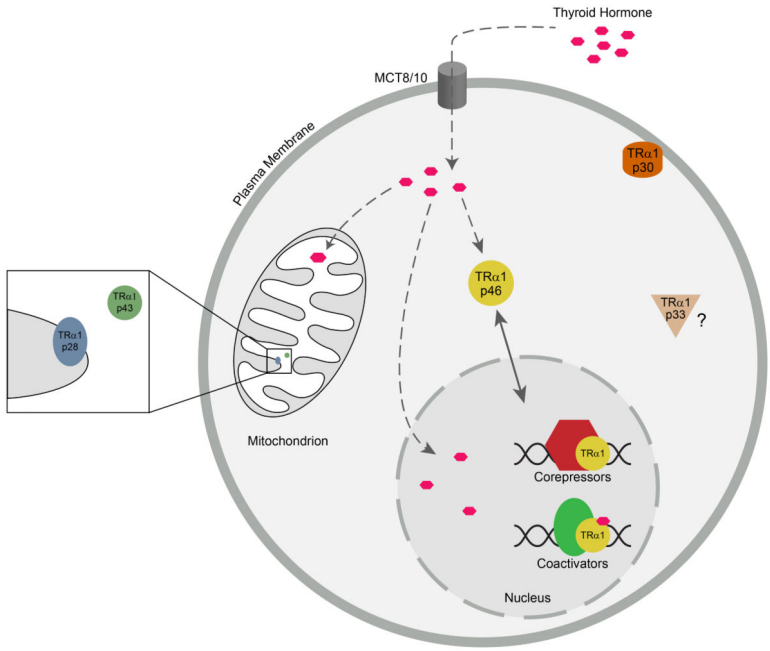

Figure 4.

Model for localization of TRα1 isoforms to the mitochondria, plasma membrane, and nucleus. TRα1 mRNA yields different forms of truncated TR by internal translation initiation. Once synthesized in the cytosol, the different forms localize to different intracellular compartments. TRα1 p28 and TRα1 p43 localize to the mitochondrial inner membrane and matrix, respectively. TRα1 p30 localizes to the inner surface of the plasma membrane, where it can bind to thyroid hormone to mediate thyroid hormone signaling. The specific localization and function of TRα1 p33 remains unknown. Full-length TRα1 (TRα1 p46) localizes to the nucleus where it modulates target gene expression in response to thyroid hormone, in association with corepressors and coactivators. Thyroid hormone enters the cell through the monocarboxylate 8 and 10 transporters (MCT8/10).

Table 1

Cellular localization and function of mammalian TRα and TRβ isoforms

Receptors	Cellular localization	Known or hypothetical function(s)
TRα (NR1A1)		
TRα1		
p46 (full-length)	Nuclear	Transcriptional activation/repression
p43	Mitochondrial matrix	Transcriptional activation/repression
p33	Unknown	Unknown
p30	Plasma membrane	Signaling cascade
p28	Mitochondrial inner membrane	Signaling cascade
TRα2	Nuclear	Possible antagonist of TR action
TRα3	Nuclear	Possible antagonist of TR action
TRΔα1	Unknown	Possible antagonist of TR action
TRΔα2	Unknown	Possible antagonist of TR action
TRα-ΔE6	Cytoplasm	Inhibitor of TR activity
TRβ (NR1A2)		
TRβ1	Predominantly nuclear	Transcriptional activation/repression
TRβ2	Predominantly nuclear	Transcriptional activation/repression
TRβ2Δ	Predominantly nuclear	Possible transcriptional regulation
TRβ3	Predominantly nuclear	Transcriptional activation/repression
TRΔβ3	Predominantly nuclear	Dominant negative antagonist
TRβ4	Predominantly nuclear	Weak antagonist of TR action