

REVIEW

Thyroid hormone receptor localization in target tissues

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Abstract

The thyroid hormone receptors, TR α 1, TR β 1 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, TR α 1 and TR β 1 shuttle rapidly between the nucleus and cytoplasm. We have identified multiple nuclear localization signals and nuclear export signals within TR α 1 and TR β 1 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 mitochondria and the inner surface of the plasma membrane.

Key Words

- ▶ thyroid hormone
- ▶ thyroid hormone receptor
- ▶ nuclear import
- ▶ nuclear export
- ▶ mitochondrial import

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Introduction

Thyroid hormone is essential for many diverse processes in nearly all vertebrate tissues, and abnormal thyroid hormone signaling underpins several human diseases (Laudet & Gronemeyer 2002, Chen *et al.* 2013, Kim & Cheng 2013, Mullur *et al.* 2014, Mondal *et al.* 2016, Mendoza & Hollenberg 2017, 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 the

regulation of mitochondrial activity (Flamant & Gauthier 2013, Pascual & Aranda 2013, Bernal 2017, Skah *et al.* 2017, Vella & 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 (Kalyanaraman *et al.* 2014, Davis *et al.* 2016).

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 & 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 *et al.* 2005, Bonamy & Allison 2006, Fernandez-Majada *et al.* 2007, Bondzi *et al.* 2011, Wang & Li 2014). The thyroid hormone receptors are remarkably dynamic proteins. Although primarily residing in the nucleus, TR α 1 and TR β 1 shuttle rapidly between the nucleus and cytoplasm, and recent characterization of TR α 1 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 (Subramanian *et al.* 2015, Roggero *et al.* 2016), 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 the understanding of the mechanism of action of TRs. In this review, we will focus on the 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 & 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, T₄) and 3,5,3'-L-triiodothyronine (T₃) are bound with three different thyroid hormone carrying proteins: thyroxine-binding globulin, transthyretin and human serum albumin (Pappa *et al.* 2015, Mondal *et al.* 2016). 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 T₃; 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 & 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 T₄ can be converted to the physiologically active hormone T₃, or inactivated via conversion to 3,3',5'-L-triiodothyronine (reverse T₃, or rT₃) within the cell. T₃ and rT₃ can be modified to form the physiologically active 3,5'-L-diiodothyronine (T₂) or the inactive 3,3'-L-diiodothyronine (3,3'-T₂), respectively, to protect tissues from excess hormone (Dentice *et al.* 2013, Orozco *et al.* 2014, Mondal *et al.* 2016). Whether T₄ is directly involved in mediating gene expression remains a subject of debate. T₄ 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 T₄ can directly modulate gene expression, dependent on the TR subtype and other cellular cofactors (Galton 2017). T₃ 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 the 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 (Ruiz-Llorente *et al.* 2011, Brent 2012, Kim & Cheng 2013).

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 α V β 3 integrin, a regulator of cell–cell and cell–extracellular matrix interactions (Martin *et al.* 2014, Mullur *et al.* 2014, Cvoro *et al.* 2016, Davis *et al.* 2016, Lin *et al.* 2016). This receptor binds T₃ and T₄ 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 & Farwell 1997), and changes in the morphology of breast cancer cells (Flamini *et al.* 2017). The α V β 3 integrin-associated receptor has two thyroid hormone-binding sites, S1 and S2, which lead to the activation of 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 twofold; 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 (Kumara-Siri *et al.* 1986, Macchia *et al.* 1992, Lee & Mahdavi 1993, Andersson & Vennstrom 1997, Zhu *et al.* 1998, Zhang & Lazar 2000). However, we and others have shown that even though TR α 1 and TR β 1 appear to be predominantly nuclear at steady state, in fact, the receptors are undergoing rapid nucleocytoplasmic shuttling in both the presence and absence of T₃ (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 TR α 1 and untagged TR α 1 detected by antibody staining show a primarily nuclear distribution at steady state (Bunn *et al.* 2001, Bonamy *et al.* 2005).

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 (Laudet & Gronemeyer 2002, Darras *et al.* 2011, Huang *et al.* 2015, Taylor & 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 TR α -encoding genes (Galay-Burgos *et al.* 2008, Darras *et al.* 2011). 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 & Habibi 2008, Politis *et al.* 2018). For example, chickens and ducks have at least three subtypes (TR α , TR β 2, TR β 0) (Bishop *et al.* 2000); zebrafish produces two TR β variants and at least three TR α isoforms that all act as functional nuclear receptors (Darras *et al.* 2011); two distinct TR α transcripts and one TR β transcript have

been isolated from the American alligator (Helbing *et al.* 2006); and the Atlantic halibut has two TR α and two TR β isoforms (Galay-Burgos *et al.* 2008). The main focus of this review is on the well-characterized mammalian receptors, in particular TR α 1 and TR β 1.

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 & Gauthier 2013, Mullur *et al.* 2014, Vella & 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 TR α 1, TR β 1, TR β 2, TR β 3 and TR β 4 (Tagami *et al.* 2010, Moriyama *et al.* 2016); and the nonreceptor TR variants that lack T₃-binding ability, TR α 2, TR α 3 and TR α - Δ E6 (Casas *et al.* 2006).

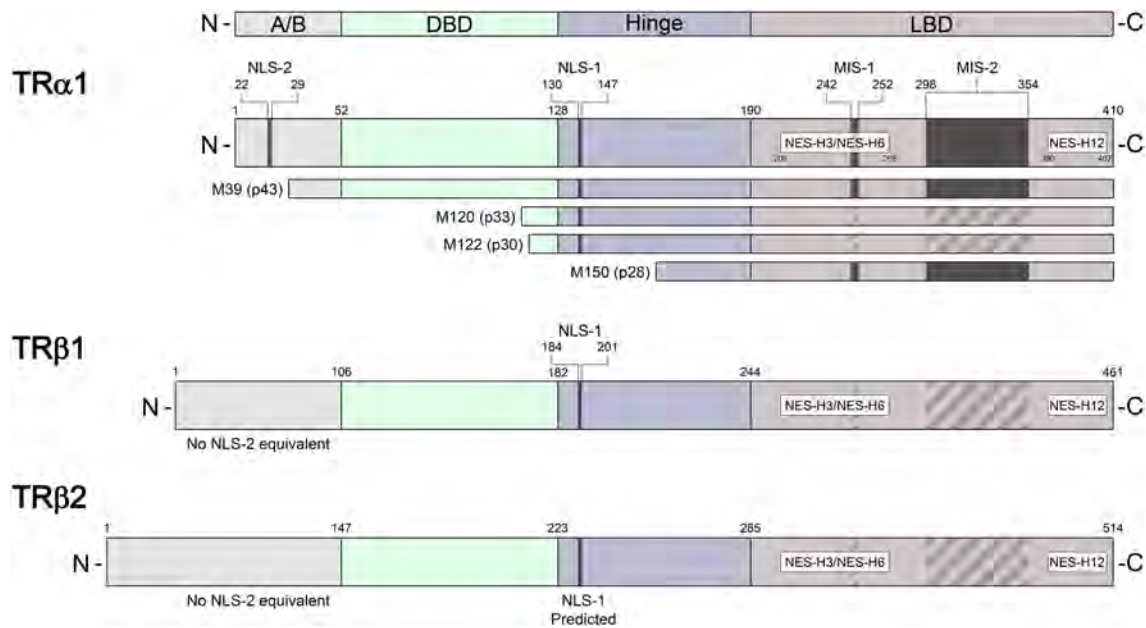
TR α 1 has the highest expression in bone, the gastrointestinal tract, cardiac and skeletal muscle and the central nervous system; TR α 2 and TR α 3 are predominant in the brain, kidney, testis, brown adipose tissue and skeletal muscle (Guissouma *et al.* 2014, Skah *et al.* 2017). TR α - Δ E6 is expressed in all tissues tested and can sequester TR α 1 in the cytoplasm (Casas *et al.* 2006). TR α 2 is found consistently in mammals but not in other species. Although a dominant negative function has been attributed to mammalian TR α 2, which is widely co-expressed with TR α 1, the physiological relevance has remained a puzzle, particularly since it is unexplained why it would be necessary for TR α 2 to counter-balance normal

TR activity in mammals but not in nonmammalian species (Vennstrom *et al.* 2010). There is recent compelling evidence, however, that TR α 2 modulates thyrotropin-releasing hormone gene expression in the hypothalamus (Guissouma *et al.* 2014). In addition, four truncated forms of TR α 1 (full-length, 46kDa) originate from alternative internal AUG translation initiation codons in TR α 1 mRNA and are named based on their molecular masses: p43 starts at the equivalent of methionine-39 (Met³⁹) in the full-length receptor, p33 starts with Met¹²⁰, p30 starts with Met¹²² and p28 starts with Met¹⁵⁰ (Kalyanaraman *et al.* 2014, Wrutniak-Cabello *et al.* 2017) (Fig. 1). Finally, other truncated forms of TR α , TR Δ α 1 and TR Δ α 2 are produced from an internal promoter in intron 7; they contain only the C-terminus of the ligand-binding domain (LBD) and are expressed in the brain, lung and gut (Chassande *et al.* 1997, Davis *et al.* 2016). TR Δ α 1 has been proposed as a candidate mediator of T₄ binding in the cytoplasm, potentially playing a role in regulating actin polymerization (Davis *et al.* 2016).

TR β 1 is most abundant in the liver, kidney and the inner ear; TR β 2 is predominant in the hypothalamus, pituitary, cochlea and retina; and TR β 4 is ubiquitously expressed, with relatively high expression in the brain and kidney (Flamant & Gauthier 2013, Hahm & Privalsky 2013, Mullur *et al.* 2014, Vella & Hollenberg 2017). Other minor isoforms of TR β 1 (52-kDa) also exist; for example, two isoforms are alternatively translated from TR β 1 mRNA, with TR β 3 (44.6-kDa) appearing to act as a functional receptor in rat (Flamant & Gauthier 2013), and TR Δ β 3 (32.8-kDa) functioning as a ligand-responsive

Table 1 Cellular localization and function of mammalian TR α and TR β isoforms.

Receptors	Cellular localization	Known or hypothetical function(s)
TR α (NR1A1)		
TR α 1		
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
TR α 2	Nuclear	Possible antagonist of TR action
TR α 3	Nuclear	Possible antagonist of TR action
TR Δ α 1	Unknown	Possible antagonist of TR action
TR Δ α 2	Unknown	Possible antagonist of TR action
TR α - Δ E6	Cytoplasm	Inhibitor of TR activity
TR β (NR1A2)		
TR β 1	Predominantly nuclear	Transcriptional activation/repression
TR β 2	Predominantly nuclear	Transcriptional activation/repression
TR β 2 Δ	Predominantly nuclear	Possible transcriptional regulation
TR β 3	Predominantly nuclear	Transcriptional activation/repression
TR Δ β 3	Predominantly nuclear	Dominant negative antagonist
TR β 4	Predominantly nuclear	Weak antagonist of TR action

**Figure 1**

Thyroid hormone receptor (TR) major subtypes and localization signals. The structural diagram (not to scale) of TR α 1, TR β 1 and TR β 2 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 TR α 1 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 TR α 1.

dominant negative antagonist (Williams 2000). In addition, an elongated form of TR β 2, termed TR β 2 Δ , 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 TR α and TR β 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 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 (Nascimento *et al.* 2006, Pawlak *et al.* 2012, Mondal *et al.* 2016, Zhang *et al.* 2018). TR α 1 and TR β 1 both contain AF-1 domains involved in the transcriptional response to

hormone; while the TR β 2 isoform, which differs from TR β 1 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 T₃ binding are complex (Schweizer *et al.* 2017). The twelfth helix contains the ligand-dependent activation domain, AF-2 (Figueira *et al.* 2011). Helix 12 forms a short pivoting structure that can adopt different conformations. In the absence of T₃, 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 T₃ may induce a hormone-dependent 'mouse-trap' mechanism (Moras & Gronemeyer 1998, Sonoda *et al.* 2008, Flamant 2016), where helix 12 rotates to swing shut and close off the pocket around T₃. 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 & 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 & 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 & Privalsky 2009). Recent X-ray crystallographic structural studies have revealed a second ligand-binding site in TR located between helices 9 and 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-kDa (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 (Tran *et al.* 2014, Cautain *et al.* 2015). They are octagonally symmetric cylindrical structures made up of proteins termed nucleoporins or Nups, which 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 & Suel 2011, Kimura & Imamoto 2014).

Our systematic characterization of NES and NLS motifs by site-directed mutagenesis has elucidated the mechanics of TR nuclear localization (Mavinakere *et al.* 2012). In-depth analysis of $TR\alpha 1$ and $TR\beta 1$ 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 $TR\alpha 1$ that is absent in $TR\beta 1$ (Fig. 1) (Mavinakere *et al.* 2012). RNAi and coimmunoprecipitation assays show that members of the importin family of karyopherins, specifically importin 7, importin $\beta 1$ and adapter importin

$\alpha 1$ 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, $TR\alpha\text{-}\Delta E6$, has a strikingly altered localization compared with $TR\alpha 1$; $TR\alpha\text{-}\Delta E6\text{-GFP}$ was shown to be predominantly expressed in the cytoplasm with minor nuclear fluorescence (Casas *et al.* 2006). In addition, $TR\beta 4$ 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 $TR\alpha 1$ 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 LBD 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

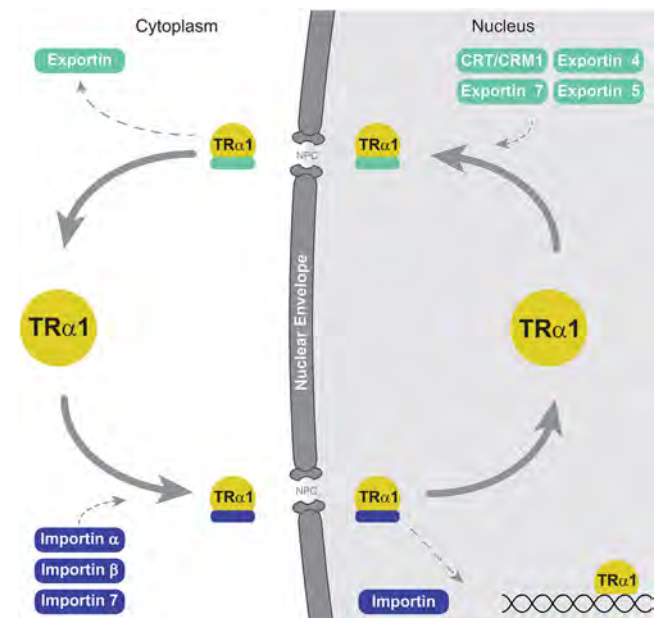


Figure 2

Thyroid hormone receptor nucleocytoplasmic shuttling pathway. The well-characterized pathway for $TR\alpha 1$ is depicted. $TR\alpha 1$ binds to specific importins in the cytoplasm, as indicated. The $TR\alpha 1$ -importin complex passes through a nuclear pore complex (NPC) embedded in the nuclear envelope into the nucleus, where the complex is disassembled and $TR\alpha 1$ binds to target genes. $TR\alpha 1$ exits the nucleus through the NPC in association with specific exportins or calreticulin (CRT)/CRM1. $TR\beta 1$ follows a similar nucleocytoplasmic shuttling pathway, but nuclear import is solely mediated by the importin $\alpha 1$ /importin $\beta 1$ complex.

influence TR export, including exportins 4, 5 and 7 (Subramanian *et al.* 2015). Not surprisingly, the two NLSs found in TR α 1 act to confer strong nuclear localization to the receptor; we hypothesize that TR β 1's small cytosolic population (Zhu *et al.* 1998, Baumann *et al.* 2001) may reflect an altered balance of NLS and NES activity (Mavinakere *et al.* 2012, Zhang *et al.* 2018) (Fig. 3). Although multiple NLS and NES motifs exist in a variety of nuclear proteins, how these multiple signals interact collectively remains unclear (Bonaldi *et al.* 2003, Mavinakere *et al.* 2012, Umemoto & Fujiki 2012, Lu *et al.* 2014, Dai *et al.* 2015, Panayiotou *et al.* 2016). 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 (Brent 2012, Bernal &

Morte 2013, Ayers *et al.* 2014, Vella & Hollenberg 2017). Thyroid hormone receptors often heterodimerize with the retinoid X receptor (RXR), expanding the range of T₃ 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 (Xu *et al.* 1999, Oberoi *et al.* 2011, Mendoza *et al.* 2017). 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 (McKenna *et al.* 1999, Soriano *et al.* 2011, Dasgupta & O'Malley 2014). 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

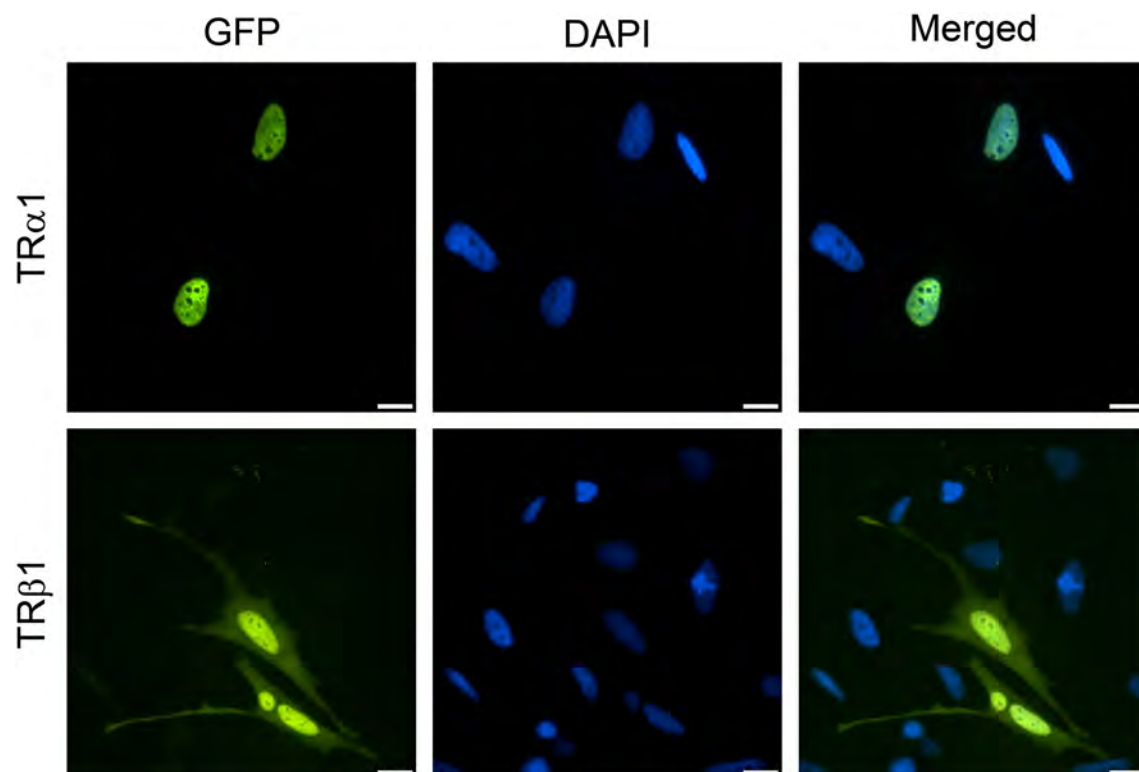


Figure 3

Distinct intracellular localization patterns for TR α 1 and TR β 1. HeLa cells transfected with expression plasmids for green fluorescent protein (GFP)-tagged TR α 1 and TR β 1 were analyzed by fluorescence microscopy after staining for DNA with DAPI to visualize the nucleus. GFP-TR α 1 predominantly localizes to the nucleus; GFP-TR β 1 also localizes to the nucleus but has a slight cytoplasmic population. Scale bar, 10 μ m.

manner (Grontved *et al.* 2015). These findings align with an earlier report that used fluorescence recovery after photobleaching (FRAP) to show that TR β 1 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 T₃ based on the availability of specific corepressors and coactivators, providing an explanation for tissue-specific responses to similar amounts of T₃ (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 T₃-dependent manner (Bernal & Morte 2013). In this instance, N-CoR1 and SMRT appear to play a role in determining T₃ sensitivity, suggesting that corepressors can be recruited to TR in the presence of T₃ (Astapova *et al.* 2011, Astapova & Hollenberg 2013, 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 T₃ (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 TR α 1 (p46) and the truncated isoforms p43, p33, p30 and p28 has revealed conflicting intracellular targeting signals within TR α 1 that can direct the proteins to the nucleus, mitochondria or the inner surface of the plasma membrane (Mavinakere *et al.* 2012, Kalyanaraman *et al.* 2014, Wrutniak-Cabello *et al.* 2017) (Figs 1 and 4). TR α 1 p43 and p28 are targeted to the mitochondrial matrix and mitochondrial inner membrane, respectively. The biological function of TR α 1 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 T₃, the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signaling cascade is activated and stimulates the 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, Kalyanaraman *et al.* 2014, Cvorovic *et al.* 2016, 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 TR α 1, TR α 2 and TR β 1 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 TR α 1 and TR α 2 with a mitochondrial marker showed a predominantly mitochondrial localization for TR α 1 proteins (Psarra & Sekeris 2008, Carazo *et al.* 2012, 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, Psarra & Sekeris 2008, Davis *et al.* 2016, Wrutniak-Cabello *et al.* 2017). The major effect of thyroid hormone on mitochondrial activity has been partially explained by reports of truncated TR α 1 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 TR β (TR β A1) localizes to the mitochondria in *Xenopus laevis* (South African clawed frog) oocytes (Saelim *et al.* 2007). TR α 1 p43 is targeted to the mitochondrial matrix, while TR α 1 p28 is targeted to the mitochondrial inner membrane (Carazo *et al.* 2012, Kalyanaraman *et al.* 2014, Wrutniak-Cabello *et al.* 2017) (Fig. 4). TR α 1 p43 displays an N-terminal deletion that lacks NLS-2, but still possesses NLS-1 in the hinge region (Fig. 1). In contrast, TR α 1 p28 displays an N-terminal deletion of the A/B domain, the DBD and NLS-1. Neither p43 nor p28 possesses a canonical mitochondrial import sequence (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,

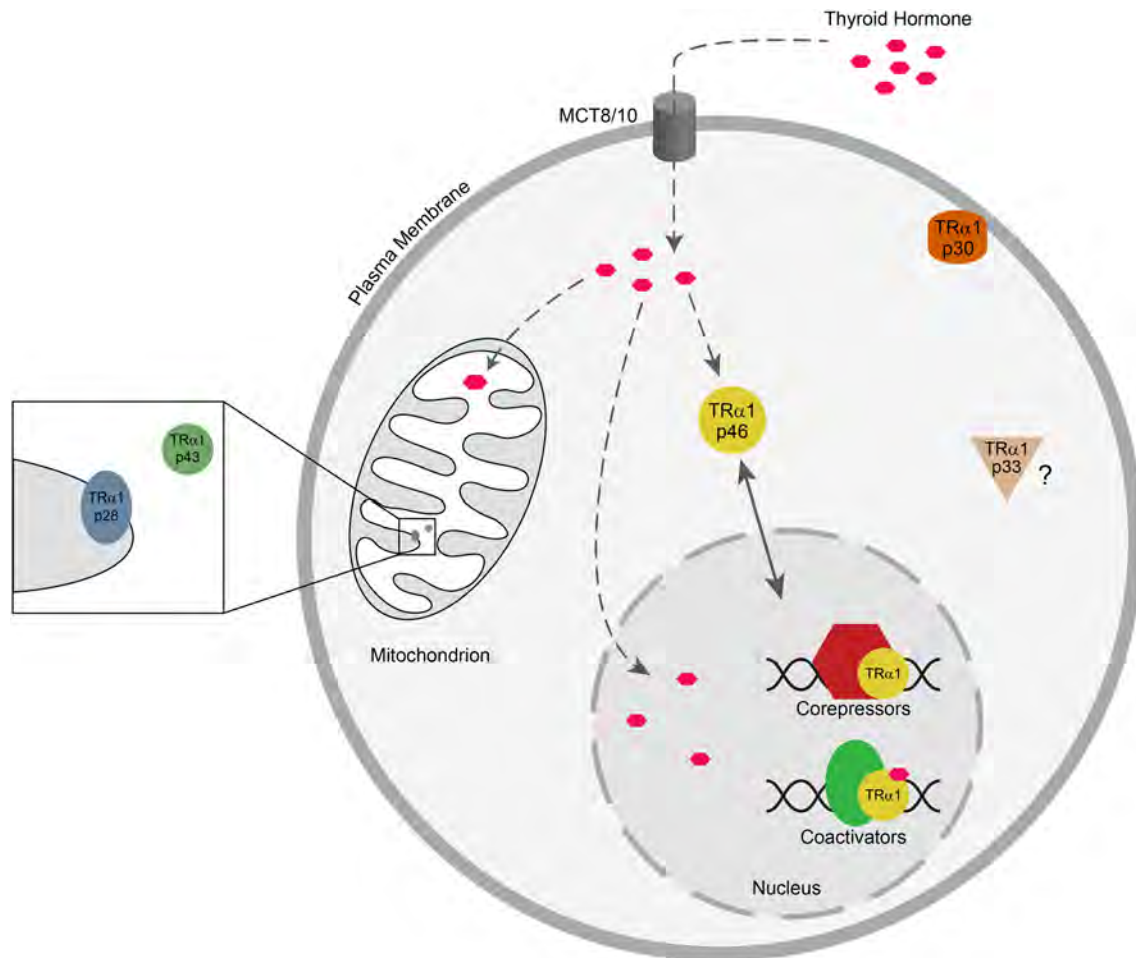


Figure 4

Model for localization of TR α 1 isoforms to the mitochondria, plasma membrane and nucleus. TR α 1 mRNA yields different forms of truncated TR by internal translation initiation. Once synthesized in the cytosol, the different forms localize to different intracellular compartments. TR α 1 p28 and TR α 1 p43 localize to the mitochondrial inner membrane and matrix, respectively. TR α 1 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 TR α 1 p33 remains unknown. Full-length TR α 1 (TR α 1 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).

spanning amino acids 242–252 of TR α 1, 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 TR α 1 (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, TR β 1 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 TR α 1 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, TR α 1 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, TR α 1 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 TR α 1 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 post-translational 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 (Rodriguez 2014, Lin *et al.* 2015, Azevedo & Saiardi 2016, Drazic *et al.* 2016). 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 (Cui *et al.* 2004, Lin *et al.* 2005, Sanchez-Pacheco *et al.* 2009, Abdel-Hafiz & Horwitz 2014, Faresse 2014). For example, the association of TR α 1 p30 with the plasma membrane is mediated by palmitoylation, a post-translational lipid modification. Consequently, it has been predicted that cysteine (Cys)²⁵⁴ and Cys²⁵⁵ 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 TR α 1 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 TR β 1 may play a role in promoting nuclear localization in serum-starved Chinese hamster ovary (CHO) cells. FLAG-tagged TR β 1 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 TR β 1 that are not conserved in TR α 1. When hormone was added, the complex dissociated, allowing PI3K activity to increase and TR β 1 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-TR β 1. As shown in Fig. 3, GFP-TR β 1 typically has a greater cytosolic population than GFP-TR α 1, and we find TR β 1 distributions ranging from whole cell to primarily nuclear. For critical analysis of the fine nuances of receptor localization, rigorous quantification of the nucleus vs 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 TR α 1 (Sanchez-Pacheco *et al.* 2009), and to K184, K188 and K190 in TR β 1 (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 TR α 1 targets the receptor for rapid proteasome-mediated degradation (Bondzi *et al.* 2011). Recently, it was reported that monoubiquitination of TR α 1 within its LBD results in a shift in the diffuse intranuclear localization of TR α 1 toward the nuclear periphery in cardiomyocytes (Wadosky *et al.* 2016). TR α 1 activity stimulates hypertrophy in cardiomyocytes, and although TR α 2 and TR β 1 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 TR α 1 *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 TR α 1 (positioned in NES-H3/H6); and at K50 (A/B domain within AF-1), K146 (DBD) and at K443 (near the NES-H12 motif) of TR β 1 (Liu *et al.* 2012, 2015, 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, TR α 1 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 TR α 1 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 T₃ binding and transactivation capacity and some exhibit dominant negative activity (Conde *et al.* 2006, Martinez-Iglesias *et al.* 2009, Rosen & Privalsky 2009, 2011, Chan & Privalsky 2010, Rosen *et al.* 2011, Kim & Cheng 2013, Lin *et al.* 2013, 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 TR α 1 to these cytosolic inclusions (Bunn *et al.* 2001, DeLong *et al.* 2004, Bonamy *et al.* 2005, Bonamy & Allison 2006, Bondzi *et al.* 2011, Takalo *et al.* 2013, Zhang

et al. 2018). 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 & Privalsky 2009, 2011). RTH syndromes exist due to mutations in the respective TR isoforms, TR α 1 and TR β 1, and the variability in symptomatic phenotype is characterized by the tissues in which these isoforms are highly expressed (Mullur *et al.* 2014, Vella *et al.* 2014, Mendoza & Hollenberg 2017, Vella & Hollenberg 2017). 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 (Parrilla *et al.* 1991, Bochukova *et al.* 2012, Dumitrescu & Refetoff 2013, Moran *et al.* 2013, 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 TR β 1 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 *et al.* 2005, Bonamy & Allison 2006).

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, TR α 1 and TR β 1 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 TR α 1 and TR β 1 nucleocytoplasmic shuttling may, at least in part, be to serve as a 'ferry boat' (Kolodkin *et al.* 2010) to increase the rate of T₃ (and possibly T₄) nuclear entry, relative to simple diffusion through the cytosol, or to circumvent localization of T₃ 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, which 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 cytoplasmic 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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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