4-2015

The Impact of the Ubiquitin-Proteasome Pathway and CRM1-Independent Exportins on Thyroid Hormone Receptor Localization and the Expression of T3-Responsive Genes

Mary Elizabeth R. Stern
College of William and Mary

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Part of the Biology Commons

Recommended Citation
https://scholarworks.wm.edu/honorstheses/194

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
The Impact of the Ubiquitin-Proteasome Pathway and CRM1-Independent
Exportins on Thyroid Hormone Receptor Localization and the Expression of T3-
Responsive Genes

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of
Science in Biology from
The College of William and Mary

Mary Elizabeth Stern

Accepted for Honors

Dr. Lizabeth A. Allison, Advisor
Dr. Shantá Hinton
Dr. Diane Shakes
Dr. Randolph Coleman

Williamsburg, VA
April 30, 2015
Abstract
Thyroid hormone is critical to the regulation of development, growth, and metabolism and is released from the thyroid gland in two forms, T₃ and T₄. T₃ movement into the cell allows it to bind thyroid hormone receptor (TR) in the cytoplasm or nucleus, and this hormone-receptor complex can regulate gene expression by enhancing or repressing transcription at thyroid hormone response elements (TREs). Alternative splicing creates six specific isoforms of TR that vary in their localization and ability to bind T₃ and TREs. The two main isoforms of TR are TRα and TRβ. The necessity of TR for hormone-dependent regulation is complicated by TR nucleocytoplasmic shuttling; proper TR shuttling dynamics allow for the nuanced patterns of gene expression observed in development, growth and metabolism. TR shuttling into and out of the nucleus is mediated by importins that recognize nuclear localization signal (NLS) motifs and facilitate nuclear import, and exportins that recognize nuclear export signal (NES) motifs and facilitate nuclear export. While TR may be partially dependent on the general CRM1/calreticulin cooperative nuclear export pathway, previous studies have suggested that TR uses an alternative exportin 5-mediated nuclear export pathway as well.

Elucidating the impact of exportin 7 (XPO7), a recently discovered exportin with a broad substrate specificity, on TR nuclear export was one of the primary objectives of this thesis research. To assess the impact XPO7 had on TR localization, transient transfections of HeLa cells with XPO7 were performed and TR distribution was examined by fluorescence microscopy. CAT ELISAs and measurement of TRE-CAT reporter gene expression under conditions of XPO7 overexpression also served as an indicator of TR presence in the nucleus. Localization data from fluorescence microscopy showed that overexpression of XPO7 shifted TR from a predominantly nuclear to either a nuclear and cytoplasmic or whole cell distribution, suggesting that XPO7 plays a central role in TR cellular localization. Analysis of transcriptional activity from CAT ELISAs proved less direct as TRα-transfected cells overexpressing XPO7 had a large amount of variability, with XPO7 overexpressing cells demonstrating both higher and lower levels of CAT reporter gene expression under control of a TRE relative to control cells, in contrast TRβ-transfected cells overexpressing XPO7 having significantly higher CAT reporter gene expression under control of a TRE relative to control cells. Another aspect of this thesis research involved assessing the possible regulatory role of the Ubiquitin-Proteasome Pathway (UPP) on T₃-responsive gene expression, as preliminary studies indicated that ubiquitinated and liganded TRα is bound to chromatin. CAT ELISAs performed under conditions of proteasome inhibition and ubiquitin overexpression proved inconclusive. Further investigation is required to better comprehend the relationship between the UPP and T₃-mediated gene expression.
# Table of Contents

**Introduction** ................................................................. 1
  Overview ........................................................................ 1
  Nuclear Receptors .................................................................. 3
  Thyroid Hormone .................................................................. 5
  Thyroid Hormone Receptor ................................................... 7
  Localization and Shuttling .................................................... 8
  Exportin 7 Structure and Function .......................................... 12
  Ubiquitination-Proteasome Pathway (UPP) and Transcriptional Regulation ................................................................. 16
  *Proteasome Structure and Ubiquitination Cascade* .................. 17
  *UPP Effects on Transcriptional Activity of different Nuclear Receptors and Histones* ......................................................... 18
  Linking TR Misregulation and Mutation to Disease .................. 24
  Thesis Objective ................................................................... 27

**Materials and Methods** ......................................................... 28
  Plasmids ............................................................................ 28
  Cell Culture ....................................................................... 29
  Transient Transfection .......................................................... 30
  Fixation, Staining, Immunofluorescence, Fluorescence Microscopy and Cell Scoring . 32
  CAT ELISAs ....................................................................... 34

**Results** ............................................................................... 38
  Exportin 7 Facilitates TR Nuclear Export ................................. 38
  Variable Impact of Exportin 7 Overexpression on TRα1-Mediated CAT Reporter Gene Expression .................................................. 43
  Increase in TRβ1-Mediated CAT Reporter Gene Expression Concurrent with Exportin 7 Overexpression ....................................... 45
  Effect of Proteasome Inhibition on TR-Mediated CAT Reporter Gene Expression ................................................................. 46
  Effect of Overexpressing Ubiquitin on TR-Mediated CAT Reporter Gene Expression ................................................................. 48

**Discussion** ......................................................................... 51
  XPO7 Facilitates TR Cytosolic Localization Shift and Augments TR-mediated Gene Expression .................................................. 51
  The Impact of Proteasome Inhibition and Ubiquitin Overexpression on Transcription ................................................................. 53

**Conclusions** .................................................................... 54

**Future Directions** ............................................................... 55

**References** ......................................................................... 57
Introduction

Overview

The cellular and organismal functions dependent on normal thyroid hormone synthesis, transport, and intracellular interaction with thyroid hormone receptor (TR) are extensive and encompass a wide range of systems in all vertebrates. Thyroid hormone is essential to development, growth, and metabolism; a more specific list of some of its functions includes the processes of neural development, pulmonary function, skeletal development and establishment of peak bone mass, various immune processes, cardiac contractility and metabolism of sugars, fats, and proteins (Brent, 2012). Regulation of thyroid hormone synthesis occurs via feedback control along the hypothalamic-pituitary-thyroid axis. Thyrotropin-releasing hormone (TRH) released from the hypothalamus causes the release of thyroid stimulating-hormone (TSH) from the anterior pituitary gland. TSH acts on the follicular cells of the thyroid gland to cause thyroid hormone release. The thyroid gland produces two forms of thyroid hormone: triiodothyronine (T$_3$) and thyroxine (T$_4$). T$_4$ is a prohormone and the major form of thyroid hormone circulating in the bloodstream; it is structurally homologous to T$_3$, with the exception of the presence of a fourth iodine. Removal of one iodine from T$_4$ creates the active hormone, T$_3$, which has a higher binding efficiency to TR than the prohormone (Brent, 2012).

TRs modulate patterns of gene expression when bound and unbound to ligand. Two main isoforms of TR, TR$\alpha$ and TR$\beta$, are essential parts of this regulatory complex, shuttling in and out of the nucleus and binding ligand. Nucleocytoplasmic shuttling is a complex process; generally only proteins with nuclear localization signal (NLS) or
nuclear export signal (NES) motifs can be transported in and out of the nucleus by importin and exportin members of the karyopherin β family through nuclear pore complexes. TR has multiple NESs: a highly conserved NES in helix 12 of its ligand-binding domain and either one or two NESs between helices 3 and 6 of the ligand-binding domain (Mavinakere et al., 2012). Both CRM1-dependent and CRM1-independent pathways are used to facilitate nuclear export of TR.

Elucidation of the mechanisms of TR nuclear export is critical to understanding many of the conditions caused by disregulation of normal nucleocytoplasmic trafficking patterns, including certain types of cancer. Atypical shuttling profiles can also impact transcription, as nuclear TR availability is a prerequisite for transcriptional regulation of T₃-responsive genes (Liu and Brent, 2010). TR availability, and thus, transcriptional activity at thyroid hormone response elements (TREs) is also dependent on rates of receptor degradation through the ubiquitin-proteasome pathway. Hypothetically, both proteasome inhibition and overexpression of ubiquitin would impact levels of gene expression at TREs. Beyond their roles in degradation, some of the enzymatic components of the ubiquitin-proteasome pathway are also capable of acting as coactivators, further complicating investigation of the impact of the ubiquitin-proteasome pathway on transcriptional regulation.

The major objectives of this thesis research were to explore the effect of overexpression of exportin 7 on the cellular localization of TR, and to investigate the transcriptional activity of T₃-responsive genes when exportin 7 is overexpressed and when the ubiquitin-proteasome pathway is manipulated.
Nuclear Receptors

Regulation of gene expression is partially facilitated through hormone signaling. Hormones travel in the blood from their site of production in the body, either freely or bound to carrier proteins dependent on their solubility, and enter a cell by a variety of methods, binding cognate nuclear receptors (NRs) at the cell surface or within the cell. The hormone-NR complex exerts control over transcription by binding to specific hormone response elements in the regulatory regions of hormonally-responsive target genes to stimulate or repress transcription.

As hydrophilic and lipophilic hormones exist and can vary in derivation from peptides, amino acids, and steroids, a variety of functionally diverse cognate NRs serve as the link between signaling hormone and transcriptional response (Brent, 2012). There are three NR classes: Class I contains receptors for steroid hormones, like progesterone and glucocorticoid receptors; Class II is the thyroid/retinoid family, including the thyroid hormone and retinoic acid receptors; and Class III comprises the orphan receptors, unique members of the superfamily, classified as receptors on the basis of their sequence homology with known receptors but lacking a known ligand (Bain et al., 2006). Class I receptors are bound to heat shock proteins and immunophilins in the absence of their ligands and are cytoplasmically sequestered. Ligand binding in Class I receptors induces nuclear translocation, homodimerization, and binding to imperfect palindromic response elements at upstream regulatory sites. Binding at these imperfect palindromic response elements is coupled to coactivator recruitment, including members of the p160 family (Xu and Li, 2003), resulting in either repression or activation of target gene transcription. Class II receptors generally heterodimerize with other Class II NRs, and bind direct, or
palindromic, response elements both in the presence and absence of ligand (Ribeiro, 1995; Aranda and Pascual, 2001; Eckey et al., 2003; Bain et al., 2006). Although binding of Class II NRs to response elements in the absence of ligand typically results in transcriptional repression, corepressors like silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) and nuclear corepressor (NCoR) also cause transcriptional repression and can be displaced by ligand binding, inducing transcriptional activation (Collingwood et al., 1999).

The basis for similarity of response in different NRs is conserved regions shared amongst all NRs: the distinctive and variable length amino-terminal domain (A/B), DNA-binding domain (DBD or C), hinge region (D), and the carboxy-terminal ligand-binding domain (LBD or E). The N-terminal region generally ranges from less than 50 to 500 amino acids and contains a transcriptional activation domain (AF-1) and multiple independent transactivation domains (Robinson-Rechavi et al., 2003; Collingwood et al., 1999). The DBD mediates NR hetero- or homodimerization and DNA-binding specificity to particular hormone response elements; a number of spatially resolved DBD structures for different NRs have revealed a conserved region containing two zinc fingers that chelate two Zn$^{2+}$ ions. Between the DBD and LBD lies a sequence-flexible hinge region containing a nuclear localization signal (NLS) that crosses into the DBD in some cases. The largest region, the LBD, is intermediate in primary sequence conservation between the DBD and hinge region. Interestingly, the LBD has highly conserved secondary structure amongst different NRs with 12 α-helices present, facilitating a wide variety of functions in addition to hormone binding, including transactivation through the AF-2 domain, dimerization through strong dimerization interfaces, NLS or NES motifs in some
cases, and often a domain with repression capabilities (Robinson-Rechavi et al., 2003; Mavinakere et al., 2012).

**Thyroid Hormone**

Thyroid hormone (TH) is integral in pathways involving growth, differentiation, and metabolism. The brain, liver, skeletal muscles and heart, and adipose tissue depend on TH for wide-ranging functions like apoptosis, neuroblast mitosis, neuronal proliferation, differentiation, lipogenesis, and lipolysis. Misregulation of endocrine signaling involving TH may have a number of deleterious effects including mental retardation, obesity, metabolic disorders, and a number of cancers, as TH is involved in numerous cross-talk pathways (Liu and Brent, 2010).

Negative feedback loops regulate TH production in follicular cells of the thyroid gland. Thyrotropin-releasing hormone from the hypothalamus travels to the anterior pituitary to cause the release of thyroid stimulating hormone (TSH) from the anterior pituitary. TSH and the TSH receptor on the follicular cell basolateral membrane facilitate iodide uptake with a sodium/iodide symporter, followed by a series of steps necessary for TH synthesis and secretion (Brent, 2012). Iodination of tyrosine residues in the glycoprotein thyroglobulin yields two forms of the hormone, active triiodothyronine ($T_3$) and the prohormone thyroxine ($T_4$). $T_3$ and $T_4$ are physically homologous with the exception of a fourth iodine on $T_4$, which is primarily removed by type 2 5′-deiodinase (D2) in humans, regulated by a ubiquitinase/deubiquitinase mechanism. $T_4$ deiodination by D2 leads to exposed lysine residues in D2: ubiquitination of these residues reduces D2 activity, and deubiquitination increases D2 activity (Sagar et al., 2008; Bianco, 2011). Type 3 5′-deiodinase (D3) provides another important regulatory pathway for TH
signaling as D3 mediates T₄ inactivation to reverse T₃ as a means of eliminating excess T₄ (Hernandez et al., 2007; Ng et al., 2010). The predominant form of TH produced by the thyroid gland is T₄ which circulates freely or bound to the carrier proteins thyroxine-binding globulin, transthyretin or albumin (Palha, 2005). Lipophilic thyroid hormone enters the cell through specific membrane transport proteins and non-amino acid specific transporters, including the monocarboxylate (MCT) family and organic anion transporters (OATPs) (Visser et al., 2011). MCT8 and MCT10 facilitate bidirectional TH transport across the plasma membrane. Human expression of MCT8 is predominantly in the brain including the cerebellar cortex and hippocampus and is present in different parts of the human hypothalamus and folliculostellate cells of the anterior pituitary. MCT10 is predominantly expressed on the basolateral membrane of the intestine, kidney, liver, muscle, and placenta. OATP1C1 has higher expression levels in the human brain and in Leydig cells of the testis and brain capillaries in rats and mice (Visser et al., 2011).

These transporters can facilitate T₄ and T₃ passage into the cell. Unliganded thyroid hormone receptor (TR) heterodimerizes with retinoid X receptor (RXR) or homodimerizes and binds to a thyroid hormone response element (TRE). RXR-TR heterodimerization occurs through their LBDs, although TR dimerization also occurs through the DBD (Kakizawa et al., 1997). This hetero- or homodimeric complex may, in some cases, stimulate gene expression at negative TREs or, more commonly, bind corepressors, such as NCoR or SMRT, to repress gene expression at positive TREs (Brent, 2012). Generally T₃ signaling and transcriptional control occurs through T₃ binding to the LBD of TR, resulting in movement of C-terminal helix 12, disruption of
corepressor binding, and promotion of coactivator binding, which then leads to recruitment of RNA polymerase II and transcription initiation (Brent, 2012).

**Thyroid Hormone Receptor**

Two different genes, THRA, located on human chromosome 17, and THRB, located on human chromosome 3, encode TRs, and produce multiple TR isoforms through alternative splicing (Nagaya et al., 1996). There are four ligand-binding isoforms: TRα1, TRβ1, TRβ2, and TRβ3. TRα1 and TRα2 are created by alternative splicing of the THRA gene, their amino acid sequences are identical until residue 370 in the LBD. TRα2 and TRα3 do not bind T₃, although alternative splicing of TRα creates additional truncated forms with varying ligand-binding ability. Different TRβ transcripts, varying in their N-terminal domains, are created through alternative promoter regions (Oetting and Yen, 2007). The different TR isoform sequences and structures lend to tissue-specific responses and variable signal transduction pathways that allow TR to function in multiple cross-talk capacities. TRα1, TRβ1, and TRβ2 have highly homologous DBDs and LBDs. The isoforms bind T₃ with similar affinity and can recognize common artificial consensus TREs *in vitro* (Chan and Privalsky, 2009). However, all of the TR isoforms have different primary sequence with resulting distinct folded conformations.

Isoform expression varies along a developmental, tissue-specific and temporal axis. TRα1 is expressed very early in development and is found in most adult tissues, whereas TRβ1 is expressed later in fetal development (Chan and Privalsky, 2009). TRα1 is primarily expressed in heart and skeletal muscle. TRβ1 has a wide expression range, while TRβ3 is principally expressed in kidney, liver, and lung. TRβ2 is the most common
isoform expressed in the retina and inner ear, while the brain seems to principally express both TRβ2 and TRα1. The viral protein v-ErbA is a mutant version of TRα1, lacking helix 12 in the LBD, preventing T₃ binding, and bearing other cancer-promoting features (Brent, 2012).

As TR is an NR, all isoforms contain the N-terminal domain and a variable AF-1 transactivation domain, the strictly conserved DBD, hinge domain, and LBD. The variable AF-1 region augments transcription activation, the DBD aids in TRE binding and dimerization, the LBD binds ligand and houses NES activity, and the hinge domain contains a bipartite NLS. TRα1 houses a second NLS in the A/B domain (Mavinakere et al., 2012).

**Localization and Shuttling**

TR is a unique NR in that it can bind target genes both in the presence and absence of ligand (Zhang and Lazar, 2000). This impacts the nucleocytoplasmic shuttling profile of TR and its role as a transcriptional regulator. Unliganded TR bound to a positive TRE generally represses transcription by recruiting corepressors like NCoR or SMRT and histone deacetylases. T₃ binding to TR induces conformational changes in TR and transcriptional activation through the release of corepressors and recruitment of coactivators like CBP/p300, pCAF, and steroid receptor coactivator-1 (SRC-1) (Kim et al., 2005). p300 and CBP are highly related histone acetyltransferases that interact with SRC-1, but also bind independently to NRs in a ligand-dependent manner (Chakravarti et al. 1996). p300 and CBP can enhance ligand-dependent transcriptional activation in collaborative interaction with SRC-1 (Smith et al. 1996). p300, CBP and SRC-1 also interact independently with PCAF (Yang et al. 1996, Torchia et al. 1997, Blanco et al.
1998) leading to the hypothesis that a large histone acetyltransferase factory might be assembled near ligand-bound receptors (Wade et al. 1997). The necessity of TR presence in forming a functional regulatory complex suggests a constitutive nuclear localization, a hypothesis which was disproven by the experimental discovery that TR shuttles rapidly between the nucleus and cytoplasm (Bunn et al., 2001). Under homeostatic conditions, TRα1 has about a 90% nuclear localization while TRβ1 has about an 85% nuclear localization.

Movement in and out of the nucleus through the water-impermeable, double-membrane, phospholipid bilayer nuclear envelope occurs through restrictive nuclear pore complexes (NPC) embedded in the envelope (Lodish, 2000). The NPC allows translocation of cargo through a central channel. Cargo moving through this central channel interacts with phenylalanine (F) and glycine (G)-repeats that form a semi-permeable barrier allowing relatively uninhibited passage of small molecules of approximately <40-60 kDa, while blocking the passage of larger molecules and preventing diffusion of macromolecules in and out of the nucleus. In addition to size, the hydrophobicity of F and G presents another impediment to the import of potential cargo. Cargo larger than 40-60 kDa must be escorted through NPCs with the help of transport proteins belonging to the karyopherin β family (Lodish, 2000). The karyopherin family is comprised of importins and exportins. The maintenance of convoluted nucleocytoplasmic trafficking patterns also depends on an asymmetric distribution of RanGTP, with higher concentrations in the nucleus and lower concentrations in the cytoplasm.

In the classical model of nuclear import, cargo bearing a short, positively-charged NLS binds a heterodimer of importin α and importin β1. Importin α acts as an adaptor,
recognizing and binding the NLS, and binds to importin β1 through an N-terminal importin β1 binding domain (IBB). Importin β1 facilitates nuclear import through direct interactions with FG repeats in the NPC (Bayliss et al., 2000). Disassociation of the nuclear import complex, enabling release of the cargo protein with an NLS, depends on RanGTP binding to importin β1 and the exportin CAS binding to importin α. The importin β1–RanGTP complex can return to the cytoplasm through the NPC. Both CAS and RanGTP are required to facilitate cytoplasmic recycling of importin α (Kutay et al., 1997). Cytoplasmic RanGAP, or Ran GTPase-activating protein, dissociates these complexes by stimulating GTP hydrolysis, converting RanGTP to RanGDP and freeing the importins for another import cycle. Cytoplasmic RanGDP is imported into the nucleus by nuclear transport factor-2 (NTF2), where RanGEF, or Ran guanine nucleotide-exchange factor, catalyses the exchange of GDP for GTP and replenishes the pool of nuclear RanGTP (Stewart, 2008). Nucleocytoplasmic shuttling is convoluted because there are a wide variety of cargoes with NLSs or NESs specific to certain members of the karyopherin β family, 20 of which are present in human cells. Furthermore, there are more cargoes with differently structured NLSs or NESs than karyopherins (Pemberton and Paschal, 2005).

Nuclear export presents a similar enigma in the sense that there seem to be many possible NESs with many possible structures binding karyopherins to create a viable export complex. One of the most well characterized exportins that TR has been shown to be partially reliant on for nuclear export is CRM1, the major karyopherin for the export of proteins out of the nucleus, as well as many RNAs. CRM1 mediates nuclear export of hundreds of proteins through the recognition of the leucine-rich NES (Dong et al., 2009).
Other well known exportins, are exportins 5 and 7, which have been demonstrated to facilitate nuclear export of microRNA precursors and p50RhoGAP/14-3-3σ, respectively (Nelson, 2013). In a typical nuclear export model, RanGTP serves as the bridge between cargo bearing an NES and an exportin. This ternary complex can move to the cytoplasm through the NPC where a cytoplasmic RanGAP stimulates GTP hydrolysis, causing the complex to disassociate, leaving the cargo in the cytoplasm. The exportin can then re-enter the nucleus to facilitate another round of nuclear export (Lodish, 2000).

Nucleocytoplasmic shuttling is crucial to the transcriptional regulation activities of TR. Certain sequences in the full-length protein, NLS and NES motifs, enable this bidirectional nuclear transport. TRα1 is unique in its possession of an additional NLS in the N-terminal domain accompanying the more ubiquitous hinge domain NLS. This N-terminal NLS is inert in v-ErbA and absent in TRβ1; however, it is necessary for efficient TRα1 nuclear import and retention. A completely nuclear localization of TRα1 in homeostatic conditions requires the N-terminal domain, DBD, and hinge domain, indicating that TR nuclear import relies on an interplay between multiple domains and NLSs. The impact of other factors like NCoR or RXR, which enhance nuclear retention of TRβ1, complicates the nuclear import model and demonstrates the intricacy of the possible interactions (Mavinakere et al., 2012). TR is thought to use both the classical nuclear import model, with an importin α1 adaptor protein and importin β1, and a non-classical model where importin 7 binds TR and facilitates nuclear import. (Roggero et al., unpublished data; Parrent, 2010)

For nuclear export, previous studies have shown that TRα1 makes use of a cooperative export pathway involving chromosome region maintenance 1 (CRM1) and
calreticulin as an adaptor protein. The dearth of leucine-rich NESs typically employed by CRM1 in TRα1 and the inability of leptomycin B (LMB) to completely inhibit nuclear export spurred investigation into alternative exportins that could facilitate TR nuclear export (Grespin et al., 2008). Multiple CRM1-independent NESs have been identified in the LBD of TR: one at helix 12 and either one or two more between helix 3 and helix 6 (Mavinakere et al., 2012). Exportin 5 plays a role in one of these CRM1-independent pathways by influencing TR localization (Nelson, 2013). Investigating the role of exportin 7 in mediating CRM1-independent export of TR is a primary focus of this thesis.

Exportin 7 Structure and Function

Exportin 7 (XPO7) was first identified by using Ran affinity chromatography on HeLa cell extracts. Immobilized RanQ69L, a GTPase-deficient mutant of Ran that stays bound to GTP regardless of the presence of RanGAP, was used to identify proteins interacting with RanGTP, specifically. SDS-PAGE and peptide sequencing revealed peptides corresponding to known transport receptors (Kutay et al., 2000). The presence of an importin β-like RanGTP-binding domain was suggested by sequence information and comparison to expressed sequence tags, which demonstrated remarkable similarity to an open reading frame from *C. elegans* that is homologous to the N-terminal regions of known importins and exportins. Using sequencing information from the *C. elegans* protein, full-length XPO7 cDNA was created. Western blotting with antibodies to a C-terminal peptide of XPO7 revealed enrichment in the RanGTP-bound fraction.

Sequence alignments place XPO7 in a distant part of the evolutionary tree, distinct from the rest of the importin β superfamily. As an example, homology between XPO7 and its closest relative, CRM1, is limited to the N-terminal RanGTP-binding
domain. However, there are multiple lines of evidence validating the inclusion of XPO7 in the importin β related nuclear transport receptor superfamily: direct interaction of XPO7 with RanGTP, its predominantly nuclear and nuclear envelope localization in living cells, direct binding of XPO7 to a nucleoporin implicated in translocation of transport receptors, and the ability to enter the nucleus without the help of any other soluble factors (Kutay et al., 2000).

The x-ray crystallographic structure of XPO7 has yet to be characterized; however, further studies have shown the highest XPO7 expression in testis, thyroid, and bone marrow. XPO7 has low expression in lung, liver, and small intestine, and no expression in thymus. There is moderate XPO7 expression in other tissues (Koch et al., 1999). XPO7 is also expressed in red blood cells and overexpressed in the cytoplasmic fraction of red blood cells in patients with hereditary non-spherocytic hemolytic anemia (von Lohneysen et al., 2011).

XPO7 mediates a general nuclear export pathway with cargoes ranging from 14-3-3σ (cytoplasmic anchor for cyclin-dependent kinases), p50RhoGAP (a GTPase-activating protein for Rho- and Rac-GTPases), STRADα (a regulator of LKB1, a serine/threonine kinase), and E2A (transcription factors of the basic-loop-helix family) (Mingot et al., 2004; Lee et al., 2010). This broad substrate specificity is especially interesting in light of the departure of XPO7-dependent NESs from a classical leucine-rich NES. XPO7-dependent NESs generally contain basic residues and folded motifs, although XPO7 has been demonstrated to facilitate export of cargoes containing a classical NES in some cases (Mingot et al., 2004; Caceres-Gorrini et al., 2014).

Conversely, an intermediate disassociation constant for RanGTP hydrolysis has led to
ambiguity in the classification of XPO7 as an importin or exportin, and the proposal that it may mediate bidirectional transport (Kutay et al., 2000). The dependence of all the cargoes listed above, with the exception of E2A, on a cytoplasmic localization for function, enhances the probability of XPO7 acting primarily as an exportin.

In accordance with the presence of multiple basic residues in XPO7-dependent NESs, E2A transcription factors are members of the basic helix-loop-helix (bHLH) protein family, characterized by high-density regions of positively charged amino acids. The nuclear presence of E2A proteins, including alternative splicing products E12 and E47, is required for their normal function as transcription factors. It has been shown that XPO7 increases E2A transcriptional activation and that E12 and XPO7 co-immunoprecipitate, further supporting the idea that positive residues increase the likelihood of cargo binding with XPO7, and that the exportin may noticeably impact the transcriptional activity of binding partners that participate directly or indirectly in transcription. The binding between E12 and XPO7 has been demonstrated as being direct; however, the interaction of E2A proteins with p300/CBP and SAGA histone acetyltransferase complex demonstrates how complicated the transcriptional regulation can become with synergistic and competitive elements influencing transcriptional activity (Lee et al., 2010).

As demonstrated by its upregulation of E2A-XPO7 genes, including the cyclin-dependent kinase inhibitor p21\(^{\text{Waf1/Cip1}}\), and cytoplasmic sequestration of negative regulator of cell cycle 14-3-3\(\sigma\), the impact of XPO7 on crucial cellular housekeeping activities may extend to cell cycle control. 14-3-3\(\sigma\) requires cytoplasmic localization to sequester the cdc2-cyclin B1 complex in the cytoplasm, which results in G2 arrest (Holm
et al., 2009); cytoplasmic p21^{Waf1/Cip1} orchestrates G1 arrest in response to low-level DNA damage, giving the cell time to repair the damage before resuming cycle progression (Lodish, 2000). Both p21^{Waf1/Cip1} and 14-3-3σ are p53-inducible genes, with 14-3-3σ acting in a positive feedback loop with p53 where 14-3-3σ expression leads to stabilized expression of p53. 14-3-3σ acted antagonistically towards Mdm2 by blocking Mdm2-mediated p53 ubiquitination and nuclear export. Other effects of 14-3-3σ on p53 stability encompass facilitation of the oligomerization of p53 and enhancement of p53 transcriptional activity. In turn, stabilized p53 can carry out its cell cycle regulatory activities (Yang et al., 2003). Thus, the direct (14-3-3σ cytoplasmic sequestration) and indirect (upregulation of E2A target gene p21^{Waf1/Cip1}) influence of XPO7 on cell cycle regulation is significant.

In addition to p53, XPO7 regulates another master tumour suppressor, LKB1, involved in cell polarity, metabolism, and cell growth. Two binding partners control the compartmental distribution of LKB1: STRADα, a direct XPO7 substrate, and Mo25. STRADα competitively inhibits LKB1 binding to importin-α/β, anchors the kinase in the cytoplasm, and facilitates nuclear export of LKB1 by serving as an adaptor between LKB1 and CRM1 or XPO7. Mo25 stabilizes the STRADα-LKB1 complex (Dorfman and Macara, 2008). LKB1 represents a major regulatory checkpoint within the cell as it is a serine/threonine kinase that directly phosphorylates and activates 13 protein kinases in the AMPK family, which includes vital metabolic sensors (Shackelford and Shaw, 2009). AMPK regulates lipid, cholesterol and glucose metabolism in specialized metabolic tissues such as liver, muscle, and adipose and it has been experimentally demonstrated that treatment with thyroid hormone increases LKB1 and Mo25 muscle content leading to
increased AMPK activation (Branvold et al., 2008). Importantly, LKB1 has a primarily nuclear distribution. The expression of STRADα, the presence of XPO7, and to a lesser degree, Mo25 facilitate stimulation of catalytic activity and cytoplasmic translocation of LKB1, which then can prompt spontaneous cell polarization in intestinal epithelial cells in the absence of cell-to-cell contacts. Nuclear-localized LKB1 mutants incapable of stable binding with STRADα have been found in cases of Peutz-Jeghers cancer syndrome, further reinforcing the impact of nucleocytoplasmic distribution on proper function (Dorfman and Macara, 2008).

The use of both XPO7 and CRM1 in STRADα nuclear export is interesting in many regards. CRM1 acts as the major STRADα exportin, binding to a short, linear, leucine-rich NES. The NES that mediates STRADα-XPO7 interaction is multipartite, requiring both N- and C-termini in addition to the classical NES used by CRM1 (Dorfman and Macara, 2008). The highest expression of XPO7 in the testis, thyroid, and bone marrow (Koch et al., 2000) suggests that the use of both exportins allows development of nuanced temporal and spatial regulation in a tissue-specific manner. This hypothesis seems more plausible when considered with the observation that XPO7 is one of only three differentially expressed genes in the process of terminal erythroid differentiation, indicating a complex role in signaling (Koury et al., 2007).

**Ubiquitination-Proteasome Pathway (UPP) and Transcriptional Regulation**

Genetic studies in yeast have shown that cells cannot survive without functional proteasomes. Proteasomes are involved in a number of vital cellular functions including transcription, the cell cycle, DNA repair, apoptosis, antigen processing, development, degradation of misfolded proteins and protein turnover (Lodish, 2000). To emphasize the
transcriptional aspect of the ubiquitination-proteasome pathway (UPP), the
transcriptional activity of most NRs is dependent on functional and active UPP
components.

*Proteasome Structure and Ubiquitination Cascade*

The 26S proteasome holoenzyme is comprised of one or two 19S regulatory
particles or caps, with ‘lid’ and ‘base’ subunits, which surround a 20S catalytic core; an
11S regulator that is involved in antigen presentation, but not protein degradation, is
present in mammalian proteasomes (Kinyamu et al., 2005). The lid of the 19S caps has
eight non-ATPase subunits and one unique subunit and is essential for degradation of
polyubiquitinated substrates. The base of the 19S caps, important in recognition of
ubiquitinated substrate by the proteasome, has six AAA ATPase subunits and three non-
ATPase subunits. ATP hydrolysis by the caps provides the energy to unfold protein
substrates and transfer them into the 20S core. The 20S core is comprised of 14 subunits,
two α rings with seven distinct subunits, α1-7, surrounding two β rings with seven
distinct subunits, β1-7, on either side in a barrel structure. The α rings do not possess
catalytic activity; however, they are thought to help facilitate the interaction between the
20S and 19S proteasome components. Three of the seven β1-7 subunits have proteolytic
active sites capable of cleaving after hydrophobic, acidic, or basic residues in a
polypeptide due to chymotrypsin-like activity, caspase-like activity, and trypsin-like
activity, respectively (Kinyamu et al., 2005). Cleavage leaves short peptides, which are
released and further degraded by cytosolic peptidases.

Proteins that enter the catalytic chamber are polyubiquitinated with at least 5
molecules of the 76-residue ubiquitin polypeptide in a process involving a specific
enzyme cascade (Lecker, 2006). Ubiquitin-activating enzyme (E1) is activated by thioester bond formation between the C-terminal end of ubiquitin and a cysteine residue on E1 in an ATP-dependent process. Activated ubiquitin is transferred to a cysteine residue on ubiquitin-conjugating enzyme (E2) by the formation of another thioester bond. In the last step in the cascade, the C-terminal glycine carboxy group of ubiquitin is attached to the ε-amino group of lysine residues or the amino group at the N terminus of the substrate protein through isopeptide bond formation catalyzed by ubiquitin ligase (E3) (Cadwell and Coscoy, 2005). Alternatively, E2 can catalyze ubiquitin transfer directly to a substrate protein through the formation of another isopeptide bond in a less common ubiquitination event. The direct interaction of the E3 enzyme with substrate confers substrate specificity to the UPP. This enzyme cascade synthesizes a polyubiquitin chain by transferring activated ubiquitin molecules to a lysine of the ubiquitin moiety previously linked to the target protein. These polyubiquitin tags are recognized by the 19S proteasome caps which unfold and transport polyubiquitinated proteins into the catalytic core for degradation. Deubiquitinase enzymes and isopeptidases in the proteasome cap remove the ubiquitin chain from the protein and hydrolyze the bonds between individual ubiquitin groups. Ubiquitinated proteins must have a specific E3 recognition motif and one or more ubiquitin-anchoring groups (Ciechanover, and Ben-Saadon, 2004).

**UPP Effects on Transcriptional Activity of different Nuclear Receptors and Histones**

In addition to the polyubiquitin tag, many unstable proteins contain PEST motifs, consisting of proline, glutamic acid, serine and threonine. These PEST motifs have a dual functionality as they can be phosphorylated and ubiquitinated, promoting degradation by
the UPP (Subramanian, 2012; Rechsteiner, 1990; Salghetti et al. 2001). This duality has important implications for transcriptional activity, as phosphorylation is thought to be involved in substrate recognition by the enzymes in the UPP and plays a vital role in the ligand-mediated stability of NRs. The overlap between sequences marking a protein for degradation in transcriptional activators and transcriptional activation domains is prominently displayed by the glucocorticoid receptor (GR) (Salghetti et al. 2001; Muratani and Tansey 2003). Normal GR is hyperphosphorylated and degraded via the UPP upon ligand-binding; however, both GR degradation and GR-mediated transcriptional activity is inhibited by mutation to its PEST motif (Kinyamu et al., 2005).

In addition to NR turnover, the proteasome plays an important role in transcription by facilitating turnover of many components of the transcription machinery, including the large subunit of RNA polymerase II (Huibregtse et al., 1997) and other associated transcription factors, like signal transducer and activator of transcription 5a (STAT5a) and Fos/Jun (Kim and Maniatis, 1996; Palombella et al., 1994). These UPP enzymes can interact with NRs and regulate their transcriptional activity in a number of ways, including: promoting degradation of negative regulators of gene transcription; recycling of factors exerting a positive influence on transcription, like NRs, general transcription factors and coactivators; disrupting the preinitiation complex enabling transcriptional elongation; or degradation of the elongating form of RNA polymerase II allowing an indefinite number of transcription reinitiations (Yan et al., 2003). Transcriptional elongation requires phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II, which, further demonstrating the association between ubiquitination and phosphorylation, causes the largest subunit to be
ubiquitinated. The phosphorylated and ubiquitinated CTD then recruits a number of elongation factors which can themselves impact the state of chromatin compaction and serve as substrates for kinases and UPP enzymes in close proximity (Gerber and Shilatifard, 2003). The phosphorylation capacity of the ATPase components of the 19S proteasome cap illustrates the close association between phosphorylation, ubiquitination, and proteasomal degradation.

In accordance with the role of proteasomes in protein turnover of components of the transcriptional machinery, chromatin immunoprecipitation has shown that proteasome subunits cluster around the promoters for hormone-responsive genes (Stenoien et al., 1999). This clustering allows various components of the UPP to act as coactivators; some notable components acting in this capacity are a ubiquitin-conjugating enzyme (UBC9), the E3 ubiquitin-protein ligases E6-associated protein (E6-AP) and receptor potentiation factor 1/reverse Spt phenotype 5 (RPF1/RSP5), and one of the ATPase subunits in the 19S cap of the 26S proteasome, yeast suppressor for Gal 1/thyroid receptor interacting protein 1 (SUG1/TRIP1). As coactivators, these UPP components possess an array of enzymatic activity influencing chromatin compaction and thus the accessibility of genes to transcriptional machinery. This characteristic is important as genome organization into compact chromatin impedes transcription (Wolffe and Hayes, 1999). This enzymatic activity includes: histone acetyltransferase (HAT), phosphorylation, histone methyltransferase, sumoylation, ADP-ribosylation, ubiquitin-conjugation, and ubiquitin-activation (Nathan et al., 2003).

It has been proposed that coactivators exert a positive influence on transcription by physically bridging activated NRs and general transcription factors. Additionally, in
concert these catalytic chromatin modifying and NR bridging activities can stimulate transcription when assembled at hormone-responsive gene promoters, through nucleosome remodeling or covalent modification of other components of the transcriptional complex (Yan et al., 2003). Reinforcing the connection between the UPP and transcription is one of SUG1’s binding partners, Cdc68, an elongation factor and component of the facilitates chromatin transcription (FACT) complex (LeRoy et al., 2000); yeast deficient in SUG1 and SUG2 are incapable of normal elongation (Ferdous et al., 2001). SUG1 binds directly to activation domains of Gal4 and viral activator VP16 (Ferdous et al., 2002) and is recruited to the estrogen receptor-responsive pS2 promoter along with E6-AP and MdM2 (Perissi et al., 2004), in just one of many examples where various UPP enzymes are recruited to NR promoters. Another ATPase subunit in the base of the 19S cap, Rpt5/TBP-1 or HIV/tat-binding protein 1, was first defined as a modulator of HIV Tat transactivation by binding to and suppressing Tat-mediated transactivation (Kinyamu et al., 2005). There is ample evidence that the enzymes of the ubiquitin cascade and proteasomal components have varying levels of necessity and sufficiency for maintaining normal transcriptional patterns.

As mentioned above, the ubiquitination system also plays a role in histone modifications. A highly conserved coactivator called SAGA (Spt-Ada-Gcn5-Acetyltransferase) that performs acetylation and ubiquitination demonstrates how differential ubiquitination patterns can regulate gene expression. The GCN5 subunit of SAGA catalyzes histone acetylation and recruits the basal transcription machinery. Deubiquitination of monoubiquitinated histones H2A and H2B can be carried out by human SAGA ubiquitin-specific protease (USP), USP22, in a deubiquitination module
with ATXN7, ATXN7L3, and ENY2. While the function of ubiquitinated histones remains to be described, ubiquitinated H2B was found throughout the genome in association with transcribed regions of highly expressed genes, and SAGA deubiquitination activity is required for full transcriptional activity mediated by NRs (Zhao et al., 2008); both of these observations and documentation of ubiquitination of histones H3 and H1 (Pham and Sauer 2000) lend credibility to the hypothesis that ubiquitination may be an influential force modulating transcription. USP22 is dependent on the presence of the rest of the components of the deubiquitination module for integration into SAGA (Lang et al., 2011) and activation and an integrated USP22 has been shown to be necessary for the transcription of target genes regulated by sequence-specific activators that require SAGA activity (Zhang et al., 2008). The deubiquitination activity of SAGA is directed more toward ubiquitinated H2B than H2A; however, cycles of ubiquitination and deubiquitination at the promoters and transcribed regions of SAGA-regulated inducible genes are required to fully activate these genes (Lang et al., 2011).

The impact of ubiquitination on NR-mediated gene expression extends beyond the deubiquitination activity of SAGA. Some reports demonstrate colocalization of ubiquitinated histones to inactive transcriptional regions. Ubiquitinated histone H2A has been found in transcriptionally inactive compartments, conflicting data suggest that it is involved in chromatin rearrangement during spermatogenesis, a process regulated by androgen and estrogen receptors (Baarends et al., 1999). Ubiquitinated histone H3 is also found in elongating spermatids (Chen et al., 1998). Monoubiquitinated histone H1 causes its release from DNA, reducing chromatin condensation and leading to gene activation (Allison, 2012). Other studies have shown that ubiquitination of histone H2B is
associated with methylation of histone H3 at specific lysine residues, which may either stimulate or repress transcription (Briggs et al., 2002). These studies are significant for NR-mediated gene transcription, as NR function can be controlled by the complex interactions between different histone modifications in cross-talk pathways (Ma et al., 2001). For instance, in *Saccharomyces cerevisiae* methylation of histone H3 at lysine residues K4 and K79 depends on ubiquitination of histone H2B, which is necessary for recruitment of proteasome subunits. Mutations in the ATPase subunits, Rpt4 and Rpt6, disrupt histone H3 methylation without altering histone H2B ubiquitination. The involvement of histone H2B ubiquitination in proteasome subunit recruitment and histone H3 methylation hints that the potential for transcriptional modulation through ubiquitination may be vast (Ezhkova and Tansey, 2004).

TR is a necessary transcription factor in the expression of genes under the control of a TRE, suggesting the possible impact that the aforementioned UPP transcriptional regulation methods could have on the expression of T3-responsive genes. Experimental data have already shown that SUG1 has a stimulatory effect on TR transcriptional activity (Fraser et al., 1997); furthermore, Rpt5 interacts with TR and enhances TR-mediated transcription cooperatively with SRC-1. Knockout of Rpt5 disrupts ligand-induced loading of TR, SRC-1, and RNA polymerase II (Satoh et al., 2009). Additionally, the E2 enzyme Ubc2 and E6-AP are necessary for TR transcriptional activation (Perissi et al., 2004), demonstrating how important functional UPP components are to TR-mediated transactivation. Conversely, proteasome inhibition significantly diminishes the ligand-induced transcriptional activity of many NRs including TR (Ramamoorthy and Nawaz, 2008). Ultimately, transcriptional activity in the
face of proteasome inhibition is NR-specific as glucocorticoid, estrogen and progesterone receptor recycling is stimulated by agonist ligand; however, estrogen and progesterone receptor transcriptional activity is impaired whereas GR is not (Nawaz et al. 1999, Wallace & Cidlowski 2001, Deroo et al. 2002). Interestingly, proteasome inhibition has a stabilizing effect on many coactivator proteins, including E6-AP, suggesting that the enzymes of the ubiquitination cascade are not exempt from destruction by the UPP.

**Linking TR Misregulation and Mutation to Disease**

The indirect and direct involvement of T₃ and TR in numerous pathways underscores how misregulation of the T₃-TR system could have a multitude of deleterious consequences affecting a wide variety of cellular processes. Resistance to Thyroid Hormone (RTH) is a prominent clinical condition associated with suboptimal T₃ nuclear action; there are many different types of receptor mutations that have been found in RTH patients. One current study has found a heterozygous missense mutation (Ala263Val) in both TRα1 and TRα2 proteins. Transcriptional function of wild-type TR is inhibited by the dominant-negative Ala263Val mutant TRα1, whereas TRα2 Ala263Val mutant function matched that of wild-type TRα (Moran et al., 2014). Another clinical investigation reported a *de novo* heterozygous nonsense mutation in the gene encoding TRα, leading to an increased affinity for the NCoR and SMRT corepressors, failed T₃-dependent corepressor disassociation, and a reduced affinity for coactivator SRC-1. These changes led to dominant negative inhibition of the wild-type receptor (Bochukova et al., 2012). Additionally, a defect in the LBD of TRβ, a glycine-arginine substitution resulting from a single guanine-cytosine replacement in the codon for amino acid 340, has also led to RTH development (Sakurai et al., 1989). Although this is one of many
characterized TRβ mutations leading to RTH. The symptoms of RTH include goiter, elevated circulating thyroid hormone levels, nonsuppressed serum TSH level, clinical euthyroidism, and tachycardia (Refetoff et al., 2007).

Similar to the genetic basis for RTH, abnormal signaling patterns and TR mutations have been documented in several forms of cancer. One form of mutant TRβ in knock-in mice, created after observing an RTH patient with the same mutation, has lost T3 binding activity and transactivation capacity and invariably develops thyroid cancer (Furuya et al., 2007). Other TRβ mutations have been seen in cases of hepatocellular carcinoma, renal cell carcinoma, and erythroleukemias (Rosen et al., 2011; Chan and Privalsky, 2010). Many of these cancers are the result of direct interaction between the TRβ mutant and the regulatory p85α subunit of phosphatidylinositol 3-kinase (PI3K); PI3K is activated during this interaction leading to increased phosphorylation of Akt and mTOR and subsequent cellular proliferation and migration (Furuya et al., 2009). In addition to the oncogenic isoform of TRα, v-ErbA, tumorigenesis in intestinal epithelial cells has been linked with TRα stimulation of transcription of the β-catenin gene (Plateroti et al., 2006).

Interestingly, CRM1 facilitates the nuclear export of many cancer-associated proteins (Nguyen et al., 2012). v-ErbA nuclear export is mediated through a CRM1-dependent pathway, and coexpression of v-Erb-A with RXR or TR leads to their cytoplasmic sequestration and abnormal patterns of T3-responsive gene expression (Bonamy et al., 2005). Survivin, a regulator of cell division in the nucleus and an apoptosis inhibitor in the cytoplasm, depends on CRM1 for nuclear export and has a primarily cytoplasmic localization in cancerous cells (Altieri, 2010; Altieri, 2008). CRM1
inhibition or disruption of the NES, resulting in nuclear retention of Survivin, increased the susceptibility of cancer cells to chemotherapy and radiation (Chan et al., 2010). CRM1 appears to participate in a regulatory feedback loop with p53, a cell cycle regulator and tumor suppressor protein (Aylon and Oren, 2011). p53 function is dependent on nuclear localization, a localization pattern primarily regulated by CRM1, to function (Cai and Liu, 2008). Some cancer cells display altered p53 localization patterns and p53 dysfunction due to increased CRM1 levels; conversely, nuclear p53 can repress CRM1 transcription (van der Watt and Leaner, 2011; Stommel et al., 1999). Another CRM1 cargo, STAT1, has a functionality mirroring p53, inducing anti-proliferative and pro-apoptotic responses in tumor cells. In addition, another member of the STAT family, STAT3, has NES homology to STAT1 and was considered a potential CRM1 cargo. Mutagenesis studies, western blotting, and immunoprecipitation indicate that XPO7 is actually the main karyopherin used for STAT3 nuclear export. Furthermore, acetylation of STAT3 on a lysine is required for interaction with XPO7 (Schroder et al., 2014). Loss of STAT3 nuclear export by silencing XPO7 can affect STAT3 downstream gene expression (Reich et al., 2006).

The overlap between exportin dysregulation, acetylation, and misregulated gene expression is suggestive of a role for the UPP in contributing to the remediation or onset of disease as well as maintaining normal transcription. Beyond the potential involvement of UPP components in exportin-related diseases, the pathway has been implicated in the development of numerous other diseases including genetic disease like cystic fibrosis and Angelman's syndrome (Schwartz and Ciechanover, 1990), and neurodegenerative diseases like Alzheimer's disease, Huntington’s disease, and amyotrophic lateral sclerosis.
Mutations and deletions to the gene for the multifunctional Parkin RING finger ubiquitin ligase have been identified as major risk factors for the onset of Parkinson’s disease. Both decreases and loss of enzyme activity are associated with the development of Autosomal Recessive Juvenile Parkinsonism. One proposal suggests that mutated Parkin has a dominant-negative effect, binding and sequestering a substrate, but stabilizing it as a consequence due to an inability to ubiquitinate the substrate (Ciechanover and Brundin, 2003). Whatever the case, the UPP represents a system in which both proteasomal and ubiquitinating enzyme defects could contribute to various disease states and, paradoxically, serve as attractive therapeutic targets for disease states unrelated to UPP components.

**Thesis Objective**

It is interesting to note that initial research classified TR as a chromatin-localized receptor (Oppenheimer and Samuels, 1983), especially since nucleocytoplasmic shuttling of TR is a critical factor in maintenance of cellular homeostasis (Bunn et al., 2001). Complex formation between T3 and TR and complex binding to TREs regulates gene expression. Conversely, TR is able to bind DNA in the absence of ligand and repress activity of its subject promoters (McKenna et al., 1999). How different factors influence the nuclear availability of TR is significant on a molecular and organismal level as misregulation can lead to a variety of thyroid-related illnesses including RTH, cancer and metabolic disease (Brent, 2012). The capacity for misregulation and mislocalization leading to disease is particularly prominent with the mutant TR variant, v-ErbA oncoprotein, which cannot mediate ligand-dependent transcriptional activation and competes for binding to TREs with normal TR-containing hetero- and homodimers (Yen...
et al., 1994). In addition, v-ErbA dimerizes with TRα and RXR, leading to their cytoplasmic sequestration (Bonamy et al., 2005).

Evidence from earlier Fluorescence Recovery after Photobleaching (FRAP) assays after knockdown of XPO7 by shRNA, suggested that XPO7 may play a role in a CRM1-independent nuclear export pathway (Subramanian et al., 2015), and preliminary experimentation yielded the finding that ubiquitinated TR bound to T3 localized to chromatin (Subramanian, 2012). This chromatin localization signifies a potent potential regulatory mechanism as components of the UPP have a widespread cellular distribution and tend to cluster around promoters for hormone-responsive genes (Stenoien et al., 1999). More complete characterization of the array of XPO7 and UPP devices for regulation of gene expression may have important implications for similar systems involving other nuclear receptors.

This thesis research examined two potential regulators of TR nuclear availability, XPO7, which was hypothesized to promote increased TR nuclear export when overexpressed and to impact T3 mediated gene expression through TR nuclear export, and the UPP, which was hypothesized to impact transcriptional activity in a variety of ways, perhaps most notably by TR degradation.

Materials and Methods

Plasmids: Exportin 7 (RanBP16), TRα1, and TRβ1 plasmids were required for transfection and introduction of exogenous receptor and exportin to ascertain what effect exportin overexpression would have on TR localization patterns. The TRα1 plasmid used throughout the course of experimentation was pGFP-TRα1, encoding rat TRα1 tagged with green florescent protein (GFP) (Bunn et al., 2001). The TRβ1 plasmid, pGFP-TRβ1,
encodes GFP-tagged, human TRβ1 (Mavinakere et al., 2012). Two expression constructs of HA-tagged human exportin 7, pMT2SM-RanBP16 and pCDNA3.1-RanBP16, were gifted to the Allison Lab from Dr. C. Smas (University of Toledo College of Medicine, Ohio).

An HA-tagged control plasmid, pCMV-HA, purchased from BD Biosciences (San Jose, CA) was used as a control for immunofluorescence, fixing, and localization assays. Chloramphenicol acetyltransferase (CAT) enzyme-linked immuno-sorbent assays (ELISA) required a CAT reporter gene linked to an empty vector as a control, and CAT reporter gene linked to a TRE enhancer for experimental conditions. The control pCAT®3-Basic Vector was supplied by Promega (Madison, WI). Experimental tk-TREp-CAT plasmid is a TRE linked with herpes simplex virus thymidine kinase (tk) promoter-CAT fusion gene reporter and was given to the Allison Lab by R. Evans (Salk Institute for Biological Studies). An HA-tagged ubiquitin plasmid, pHA-Ubiquitin, gifted to the Allison Lab by K. Fryrear (EVMS), was also used in CAT ELISAs.

Plasmids were purified with Qiagen midi- and mini-prep kits after plasmids were transformed into E. coli DH5α cells and given time to multiply in culture. Purification was performed in accordance with the Qiagen-plasmid purification protocol and plasmid purity and yield was assessed with a NanoDrop ND-1000 spectrophotometer.

Cell Culture: HeLa cells (ATCC #CCL-2) were cultured in Nunc filter-capped flasks and Gibco Minimum Essential Medium (MEM) with Invitrogen Fetal Bovine Serum (FBS) to 70-90% confluency, at which point, the extracellular matrix proteins enabling adherence to the flask surface were digested with Trypsin serine protease. Trypsinization involved aspiration of MEM, then washing the HeLa cells in approximately 15 mL Dulbecco’s-
Phosphate Buffered Saline (D-PBS)(0.10 g KCl, 0.10g KH₂PO₄, 4.00 g NaCl, and 1.08 g Na₂PO₄•7 H₂O in 500 mL), taking care to add D-PBS to the side of the flask opposite cell adherence. Following the D-PBS wash, flasks underwent two successive incubations. The first incubation occurred at room temperature in approximately 2 mL 0.25% Trypsin which was then aspirated. The second two minute incubation occurred in a ThermoScientific incubator at 37°C temperature, 98% humidity, and 5% CO₂. Dislodged viable HeLa cells had a rounded morphology and appeared distinct from the flask surface. These dislodged cells were resuspended in 8 mL of MEM. A portion of the resuspended cells were transferred to six-well (Costar) or 100 mm vented plates. Seeding required extraction of 20 µL of this resuspension for cell counts with a hemocytometer. Round Coverslips for Cell Growth (Fisher) were added to each well in the six-well plates and seeded at a density of 2.4-3.2 x 10⁵ cells per well in 2 mL MEM. A similar procedure was used to seed 100 mm plates; resuspended cells were seeded at approximately 6.0-7.0 x 10⁵ cells per plate in a total volume of 10 mL MEM. Cell vessels were incubated in the ThermoScientific incubator at the conditions listed above. Tissue culture work was performed in a Labconco Cabinet following Biosafety level II protocol.

**Transient Transfection:** Transient transfection was used to overexpress proteins and assess their localization patterns and expression levels through fixation, staining and immunofluorescence and CAT ELISAs. Transfections were performed on HeLa cells that had reached 70-80% confluency in the wells of a six-well plate or a 100 mm plate. In conditions requiring the overexpression of only one plasmid, 2 µg of one type of plasmid DNA was diluted in 250 µL Invitrogen Opti-MEM Reduced Serum Medium (Opti-MEM). For cotransfections, 1 µg of each type of plasmid DNA was diluted in 250 µL
Opti-MEM. Transfections can be facilitated through electrical or chemical means by use of electroporation or calcium chloride, in this case, Invitrogen Lipofectamine 2000 (Lipo) was used to facilitate transfection through the formation of liposome-DNA complexes taken up by endocytosis. 4 µL Lipo was diluted in 246 µL Opti-MEM; this Lipo dilution and the plasmid dilutions were incubated separately at room temperature for 5 minutes and then combined and incubated at room temperature for another 20 minutes. These transfection sets with a total volume of 0.5 mL were added to each well for six-well plate transfections and fixation, staining, and immunofluorescence assays. Transfection mixtures were incubated in the six-well plates for 6 hours in the ThermoScientific incubator at the conditions listed above before the transfection medium was aspirated and replaced with 2 mL MEM in each well. Transfections in preparation for exportin 7 CAT ELISAs called for a slightly different protocol in which 3 plasmids were used in each transfection and 3.33 µg of each type of plasmid DNA, 9.99 µg of plasmid DNA total, was diluted in 1.25 ml Opti-MEM, and 20 µL Lipo was diluted in 1.25 ml Opti-MEM. After the separate and combined incubations as described above, the transfection volume of 2.5 mL was added to each plate, already containing 10 mL MEM. This transfection procedure was mirrored in proteasome inhibition CAT ELISAs; however, there were only two plasmids used in the proteasome inhibition transfection, meaning transfections consisted of 6.66 µg each of two types of plasmid DNA. Transfections in preparation for ubiquitination CAT ELISAs alternated between transfection with two plasmids for certain plates, requiring 6.66 µg of plasmid DNA, and three for others, requiring 9.99 µg of plasmid DNA.
Fixation, Staining, Immunofluorescence, Fluorescence Microscopy and Cell Scoring: To give the transfection procedure sufficient time for transcription and translation of episomal plasmid, the transfected samples were incubated in the ThermoScientific incubator for approximately 24 hours before being fixed and stained. Before fixation and staining, seeded coverslips in each well of a six-well plate were checked for adequate confluence. With the exception of initial medium aspiration, which was performed in the Labconco Cabinet, the remainder of the procedure was performed outside tissue culture conditions. Following media aspiration, each well, and thus each seeded coverslip, was washed three times with 2 mL 1 X D-PBS for 15 seconds. Cells were subsequently fixed in a Fisher Hamilton fume hood for 7 minutes in 3.7% formaldehyde solution, diluted in 1 X D-PBS. Following the fixation period, the formaldehyde solution was pipetted out of each well in the fume hood and disposed of in the appropriate waste container. Returning to the lab bench, cells were rinsed three times for 5 minutes each in 2 mL 1 X D-PBS. After aspirating the last D-PBS wash from the wells, cells were permeabilized by one five minute wash in 0.2% Triton-X-100 solution, diluted in 1 X D-PBS, which was subsequently aspirated from each well. The cycle of three 5-minute washes in 2 mL 1 X D-PBS was repeated and the last D-PBS wash was left in each well to ease coverslip removal and inversion with tweezers on a 30 μL drop of 1:50 diluted primary antibody solution composed of 1 X D-PBS, Invitrogen 1.5% Normal Goat Serum, and the antibody. The primary antibody used in this case was a rabbit polyclonal antibody to the HA tag (Abcam #ab9110), as both the control and exportin plasmid encode HA tags. The inverted coverslips were incubated on the primary antibody solution for one hour in a humidified chamber, consisting of parafilm placed on top of a wet paper towel in a
Tupperware container. Tweezers were used to replace coverslips in the six-well plate cell-side up and subjected to another cycle of three 5-minute washes in 2 mL 1 X D-PBS. A second 45 minute incubation in a dark, humidified chamber on a secondary antibody solution was then performed by removing coverslips from wells with tweezers and inverting them on a 45 µL drop of a 1:100 secondary antibody dilution. The secondary antibody dilution was composed of secondary antibody (Vector Laboratories, T1-1000, Texas Red anti-rabbit IgG [H+L]), 1 X D-PBS, and 1.5% normal goat serum. Following the second incubation, coverslips were removed from the humidified chamber, and replaced, cell-side up in the six-well plate. Another cycle of three 5-minute washes in 2 mL 1 X D-PBS was performed. Coverslips were removed from the wells and one edge was placed on a Kimberly-Clark KimWipe to remove excess liquid before mounting in an 8 µL drop of Fluoro-Gel II (Electron Microscopy Sciences) with 4′, 6-diamidino-2′-phenylindole (DAPI) (2 µL of 0.25 µg/µL DAPI stock per 1 mL Fluoro-Gel II) on a Fisherfinest, Fisher Scientific glass slide. These slides were labeled and stored in a slide box at 4°C for about 24 hours prior to scoring via fluorescence microscopy to allow the mounting medium to harden.

These slides were blinded and then scored with an inverted Nikon Eclipse TE2000E fluorescence microscope using the inverted 60 X Oil Immersion objective. Counter stain DAPI allows stained nuclei to be seen with the UV-2E/C light filter block. Transfection with GFP-tagged TR required use of the FITC B-2E/C light filter block to excite (Blue Excitation) the green fluorescent tag and observe intracellular TR localization. Confirmation of cotransfection with GFP-tagged TR and HA-tagged exportin 7 was provided by observing the cells under the Red Excitation TRITC T-2E/C
light filter block. Cells were photographed with a Nikon A1Rsi microscope Ti-E-PFS camera, with a 40X water objective. NIS-Elements 2.30 software and Adobe Photoshop CS were used to refine images and Microsoft Excel was used to construct bar graphs.

At least 100 cells per slide were counted and classified into one of three TR localization categories in a minimum of three replicate trials: Nuclear greater than cytoplasmic (N > C), cytoplasmic and nuclear (N + C), and cytoplasmic greater than nuclear (C > N). Post-scoring, the blinded designators for each slide were removed and statistical tests including averages, standard deviation, standard error, and unpaired Student’s t tests with the two tailed P value were performed with Microsoft Excel.

CAT ELISAs: Chloramphenicol acetyltransferase (CAT) enzyme-linked immuno-sorbent assays (ELISA) are a convenient method for quantifying TRE activity by measuring the amount of CAT protein present in lysates from cells transfected with CAT reporter gene under control of a TRE. As CAT is a bacterial enzyme without a eukaryotic equivalent, its presence in transfected HeLa cells indicates enhancer activity, which for TREs, is dependent on TR availability. Thus CAT protein levels are indicative of TR presence in the nucleus. Over the course of experimentation, three separate sets of CAT ELISAs (Kit purchased from Roche Applied Sciences) were performed to explore different aspects of TR transcriptional availability and activity. The first set of CAT ELISAs was performed to assess how exportin 7 impacted TR localization. Preliminary data indicated that ubiquitinated TR was bound to chromatin in the presence of T₃ (Subramanian, 2012), the second and third sets of CAT ELISAs sought to evaluate the relevance of ubiquitination to T₃-mediated gene expression via treatment of transfected cells with proteasome inhibitors and lastly, transfection with ubiquitin plasmid to induce ubiquitin
overexpression. The transfection procedure varied between different CAT ELISA sets and will be addressed in the following passages.

For the exportin 7 CAT ELISAs, each experiment replicate required six 100 mm plates seeded with 6.0-7.0 x 10^5 cells per plate in 10 mL MEM. Transfections were performed approximately 24 hours after seeding with the plates incubated in a ThermoScientific Incubator (conditions listed above) in the intervening time. There were two groups of control transfections and one experimental transfection with two plates in each group, a plate without additional T₃ added and a plate to which exogenous T₃ had been introduced. The first control group of plates was transfected with 3.33 µg of each of the following plasmids according to the procedure described under “Transient Transfection”: pCAT®3-Basic Vector, pCMV-HA, and pGFP-TRα1. The second control group of plates was transfected with 3.33 µg of each of the following plasmids: tk-TREp-CAT, pCMV-HA, and pGFP-TRα1. The experimental group was transfected with 3.33 µg of each of the following plasmids: tk-TREp-CAT, pMT2SM-RanBP16, and pGFP-TRα1.

The second CAT ELISA set required the same procedure (see “Transient Transfection”); however, there were only four plates used in each experiment replicate in the second CAT ELISA set. All plates were initially transfected only with 3.33 µg pGFP-TRα1 and 3.33 µg tk-TREp-CAT.

The third CAT ELISA set had an identical transfection procedure to the one implemented in the second set; however, 3.33 µg of pHA-Ubiquitin was also added to two of the four plates in each experiment replicate.
Each set of CAT ELISAs was incubated for 6 hours in the ThermoScientific Incubator (conditions listed above) before the media containing the Opti-MEM was removed and replaced with 10 mL MEM per plate. At 12 hours post-transfection, the media was aspirated and replaced with 10 mL 10% Charcoal-Dextran Stripped FBS (Life Technologies) in MEM (CD-Stripped MEM) per plate. During the second media change, 10 µL of 100 µM T3 was added to one of the plates in each group from the first CAT ELISA set; T3 was added to half of the plates in the second and third CAT ELISA sets during the second media change. Additionally, 10 µL of 100 µM MG132, a potent proteasome inhibitor, was added to half of the plates in the second CAT ELISA set at this time. MG132 was dissolved in ethanol. To control for this addition, 10 µL 100% ethanol was added to plates that did not receive MG132. After the second media change, the plates were incubated in the ThermoScientific incubator (conditions listed above) for 12 hours before the CAT ELISA procedure, identical for each CAT ELISA set, was begun.

At 24 hours post-transfection, medium was aspirated from each plate in the Labconco Cabinet. Plates were washed three times with 5 ml of pre-chilled 1 X D-PBS, taking care to add buffer to the side of the plate to avoid disturbing the cells. Cell lysis involved adding 1.0-1.5 mL 1 X Lysis Buffer to each plate, making sure it washed over the entire plate, and a 30 minute incubation on ice. Cells were then scraped off of each plate using a Fisherfinest, Fisher Scientific sterile cell scraper, and the lysed cells suspended in lysis buffer were pipetted into 2.0 mL Eppendorf tubes, incubated at room temperature for 7 minutes and centrifuged for 10 minutes at 14000 x g and either 4°C or 20-25°C. After centrifugation, the supernatant was transferred to 1.5 mL Eppendorf tubes and protein concentration was determined with the Pierce BCA Protein Assay. Working
Reagent and Bovine Serum Albumin (BSA) Standards were prepared fresh for each CAT ELISA trial. Standards had BSA concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500, 2000 μg/mL. 25 μL of each standard or unknown sample was pipetted into a microplate (Nunc) well. 200 μL of Working Reagent was then added to each well and the microplate was mixed gently for 30 seconds. The microplate was covered with parafilm and incubated at 37°C for 30 minutes, cooled to room temperature, and the absorbance of each standard or unknown was measured at 560 nm with a microplate reader (GloMax Multi-Detection System, Promega). The protein concentration of the samples was determined by interpolation from the absorbance line created by the BSA samples and blank. Protein concentrations for each sample were standardized and a CAT Enzyme working dilution was prepared fresh for each experiment. Preparing the working dilution involved dilution of a 40 μL CAT stock solution in 3.96 mL sample buffer to create working dilution samples with CAT concentrations of 0, 0.125, 0.25, 0.5, and 1.0 ng/mL.

200 μL of CAT standards, a blank (3,3′,5,5′-Tetramethylbenzidine or TMB) (Sigma-Aldrich), or cell extracts were pipetted into each well and the plate was covered with foil. Two wells were filled with blank and two with each standard or cell extract. The microplate, with anti-CAT antibodies adsorbed onto the base of each microplate well, was incubated for one hour at 37°C before solution removal and rinsing of each well five times for 30 seconds each with 250 μL 1 X Washing Buffer. 200 μL anti-CAT-digoxigenin (Anti-CAT-DIG) working dilution was then added to each well, the microplate was covered in foil and another hour-long incubation at 37°C was performed followed by anti-CAT-DIG solution aspiration and an identical wash cycle to the one performed after the first hour-long incubation: rinsing of each well five times for 30
seconds each with 250 μL 1 X Washing Buffer. 200 μL anti-DIG-peroxidase (POD) working dilution was then added to each well, and the microplate was covered in foil and incubated for one hour at 37°C. Another wash cycle ensued, and after the last wash was removed 100 μL TMB was pipetted into each well and incubated at room temperature for approximately 15 minutes, until the pronounced color change was complete. The reaction was stopped by adding 100 μL 2M sulfuric acid to each well and the absorbance at 450 nm was read against TMB reagent blank with a GloMax Multi-Detection System (Promega). The absorbance measurements were averaged and used to determine relative CAT expression in each sample with Microsoft Excel. Further statistical testing was performed using Microsoft Excel (described in Fixation, Staining, Immunofluorescence, Fluorescence Microscopy and Cell Scoring).

**Results**

**Exportin 7 Facilitates TR Nuclear Export**

Data from previous fluorescence recovery after photobleaching (FRAP) experiments indicated that exportin 7 (XPO7) may be involved in TR nuclear export, as XPO7 knockdown resulted in slower TRα1 shuttling dynamics as compared to the control (Subramanian et al., 2015). Transient transfection, immunofluorescence assays, and fluorescence microscopy were performed to better understand the impact of XPO7 on nucleocytoplasmic shuttling of TR. Two sets of control cells for both TRα1 and TRβ1 were used in the transfection assays; the first control set was only transfected with either pGFP-TRα1 or pGFP-TRβ1 to demonstrate the baseline intracellular distribution of TR. The second control set was cotransfected with either pGFP-TRα1 or pGFP-TRβ1 and pCMV-HA to ensure that the HA-tag alone did not have an effect on TR distribution. The
experimental sets were cotransfected with either pGFP-TRα1 or pGFP-TRβ1, and an expression plasmid for HA-tagged XPO7 either in the vector pMT2SM or pCDNA3.1 to ensure that the particular clone used did not affect expression levels or function of XPO7. After immunostaining with anti-HA antibodies to detect the HA tag and confirm cotransfection, successfully cotransfected cells were scored for TR distribution by fluorescence microscopy. Three localization categories were used in scoring: nuclear greater than cytoplasmic (N>C), cytoplasmic and nuclear (N+C), and cytoplasmic greater than nuclear (C>N). Using scoring data from at least four experimental replicates, overall a significantly greater percentage of cells cotransfected with XPO7 showed a more cytoplasmic distribution of TR (Figure 1A and B).

The averages from five replicates performed with XPO7 in the pMT2SM vector showed a statistically significant shift from 2.4% (±1.5%) of cells in the C>N category for the second control set, transfected with pGFP-TRα1 and pCMV-HA, to 28.2% (±5.4%) of cells in C>N category for the experimental set, transfected with expression plasmids for GFP-TRα1 and HA-tagged XPO7 (P = 0.0017) (Figure 1A). These same assessments for TRβ1 showed a significant shift from 4.2% (±1.7%) of cells in the C>N category for the second control set, transfected with pGFP-TRβ1 and pCMV-HA, to 41.4% (±3.7%) of cells in the C>N category for the experimental set, transfected with expression plasmids for GFP-TRβ1 and HA-XPO7 (P < 0.001). Conversely, there were significant shifts out of the N>C localization category in cotransfected cells expressing XPO7. Cells overexpressing XPO7 and GFP-TRα1 had 39.6% fewer cells in the N>C localization category than cells overexpressing only GFP-TRα1 (P < 0.001); a 30.8%
reduction in the N>C category was observed from cells overexpressing GFP-TRβ1 to cells overexpressing GFP-TRβ1 and XPO7.

The averages from four transfection and immunofluorescence replicates performed for XPO7 in the pCDNA3.1 vector showed a shift from 3.0% (±1.8%) of cells in the C>N category for the first control set, transfected with pGFP-TRα1, to 20.8% (±2.6%) of cells in C>N category for the experimental set, transfected with expression plasmids for GFP-TRα1 and HA-XPO7 (P = 0.0012) (Figure 1 B). These same measurements for TRβ1 showed a statistically significant shift from 15.5% (±1.9%) of cells in the C>N category for the first control set, transfected with pGFP-TRβ1, to 29.8% (±2.0%) of cells in the C>N category for the experimental set, transfected with expression plasmids for GFP-TRβ1 and HA-XPO7 (P = 0.0013). A similar shift out of the N>C category in cells overexpressing XPO7 in the pCDNA3.1 vector and GFP-TRβ1, compared to control cells only overexpressing GFP-TRβ1 was observed; there was a 23.5% reduction in cells classified N>C from cells overexpressing GFP-TRα1 to cells overexpressing GFP-TRα1 and HA-XPO7 and a 21.5% reduction for GFP-TRβ1 transfected cells in these categories. Taken together, these results provide evidence that exportin 7 plays a key role in facilitating TR nuclear export.
TR Localisation (Percent of Cells)

- GFP-TRα
- GFP-TRα and CMV-HA
- GFP-TRα and HA-XPO7
- GFP-TRβ
- GFP-TRβ and CMV-HA
- GFP-TRβ and HA-XPO7

Legend:
- N>C
- C+N
- C>N
Figure 1 A and B. TR Localization with Exportin 7 in pMT2SM (A) and pCDNA3.1 (B) Transfection: Percent of cells in each localization category for transfection with XPO7 in two different vectors. Localization categories were: Nuclear>Cytoplasmic (N>C), Cytoplasmic and Nuclear (C+N), and Cytoplasmic>Nuclear (C>N). There were four sets of controls for each transfection: cells overexpressing pGFP-TRα1 or pGFP-TRβ1 alone, or cells overexpressing pGFP-TRα1 or pGFP-TRβ1 as well as pCMV-HA. Experimentally transfected cells overexpressed pGFP-TRα1 or pGFP-TRβ1 as well as pMT2SM-HA-XPO7 (A) or pCDNA3.1-HA-XPO7 (B). Error Bars are Standard Errors of the Mean. For Exportin 7 in pMT2SM transfections, n = 5 replicates. For Exportin 7 in pCDNA3.1 transfections, n = 4 replicates. A minimum of 100 cells were scored per slide.
Variable Impact of Exportin 7 Overexpression on TRα1-Mediated CAT Reporter Gene Expression

TR nuclear localization, complex formation with T₃, and binding of the hormone-receptor complex with a TRE is a necessity for transcription of T₃-responsive genes (Liu and Brent, 2010). If XPO7 provided a CRM1-independent export pathway for TR, XPO7 overexpression would be predicted to decrease expression of T₃-responsive genes. This hypothesis was tested by performing CAT ELISAs, transfecting cells with a CAT reporter gene under positive control of a TRE, and quantifying CAT protein levels from whole cell lysates. Two sets of control cells were transfected with pCAT®3-Basic Vector, pCMV-HA, and pGFP-TRα1; another two sets of control cells were transfected with tk-TREp-CAT, pCMV-HA, and pGFP-TRα1. Two sets of experimental cells were transfected with tk-TREp-CAT, HA-XPO7, and pGFP-TRα1. One of each of the transfected cell sets was incubated with 100 nM T₃ for 12 hours and then lysed to quantify CAT protein levels. Absorbance measurements from cell sets that had been incubated with T₃ were divided by absorbance measurements from the cells in the set that had not been incubated with T₃ to obtain relative CAT protein expression levels.

Comparing relative CAT expression of experimental cells overexpressing XPO7 to the controls yielded variable results. Out of three replicates and four total trials, CAT protein levels were lower in cells overexpressing XPO7 relative to control cells also transfected with CAT reporter gene under positive control of a TRE in three trials. In one of the trials, the experimental set had almost double the level of CAT protein relative to the detected level of CAT protein in the control cells transfected with CAT reporter gene under positive control of a TRE. It seems unlikely that the ‘outlier’ assay was invalid or
false due to similar variability in results when a senior lab member performed the same assays\(^1\). Averaging the data from all trials and replicates led to a compilation demonstrating that XPO7 transfected cells had only slightly less CAT reporter gene expression, presumably by facilitating TR\(\alpha\) nuclear export, but this trend was not statistically significant (Figure 2). The variability led to large standard deviation and standard error of the mean; even in the three trials with higher CAT expression in control cells, the quantified protein level was variable, ranging between 1.11 and 2.08 times higher CAT protein levels in control cells relative to experimental cells \((P = 0.793)\).

Figure 2. The Effect of Exportin 7 Overexpression on CAT Reporter Gene Expression in TR\(\alpha\) Transfected Cells: The first bar represents relative CAT protein expression in control cells transiently transfected with pCAT®3-Basic Vector (EV), pCMV-HA (HA), and pGFP-TR\(\alpha\)1 (GFP-TR\(\alpha\)1). The second bar represents relative CAT protein expression in control cells transiently transfected with tk-TREp-CAT (pCAT), pCMV-HA (HA), and pGFP-TR\(\alpha\)1 (GFP-TR\(\alpha\)1). The third bar represents relative CAT protein expression in control cells transiently transfected with pMT2SM-RanBP16 or pCDNA3.1-RanBP16 (XPO7), tk-TREp-CAT (pCAT), and pGFP-TR\(\alpha\)1 (GFP-TR\(\alpha\)1).

\(^1\) Rose C. Dziedzic
Relative CAT protein expression levels were determined by dividing absorbance measurements from plates to which T₃ had been added from plates to which no T₃ had been added; plates in the same set were transfected with the same plasmids, their treatment only differed in T₃ addition. The error bars represent the Standard Error of the Means. n = 4 replicates.

**Increase in TRβ1-Mediated CAT Reporter Gene Expression Concurrent with Exportin 7 Overexpression**

Two sets of control cells were transfected with pCAT®3-Basic Vector, pCMV-HA, and pGFP-TRβ1; another two sets of control cells were transfected with tk-TREp-CAT, pCMV-HA, and pGFP-TRβ1. Two sets of experimental cells were transfected with tk-TREp-CAT, XPO7, and pGFP-TRβ1. One of each of the transfected cell sets were incubated with 100 nM T₃ for 12 hours and then lysed to quantify CAT protein levels. The same procedure was performed with pGFP-TRβ1 transfected cells to obtain relative CAT protein expression levels as with pGFP-TRα1 transfected cells. Comparing relative CAT expression of experimental cells overexpressing XPO7 to the controls demonstrated consistently higher levels of CAT expression in experimental cells overexpressing XPO7. Out of four replicates and four total trials, CAT protein levels were significantly higher in cells overexpressing XPO7 relative to control cells also transfected with CAT reporter gene under positive control of a TRE. Consistency in CAT expression across the trials for control and experimental cell sets resulted in low standard error of the mean and low standard deviation. Combining the data from all trials yielded an average 1.89 fold increase in CAT expression in XPO7 transfected cells relative to control cells transfected with the TRE-CAT reporter gene plasmid (P = 0.00846) (Figure 3).
Figure 3. The Effect of Exportin 7 Overexpression on CAT Reporter Gene Expression in TRβ1 Transfected Cells: The first bar represents relative CAT protein expression in control cells transiently transfected with pCAT®3-Basic Vector (EV), pCMV-HA (HA), and pGFP-TRβ1 (GFP-TRβ1). The second bar represents relative CAT protein expression in control cells transiently transfected with tk-TREp-CAT (pCAT), pCMV-HA (HA), and pGFP-TRβ1 (GFP-TRβ1). The third bar represents relative CAT protein expression in control cells transiently transfected with pMT2SM-RanBP16 or pCDNA3.1-RanBP16 (XPO7), tk-TREp-CAT (pCAT), and pGFP-TRβ1 (GFP-TRβ1). Relative CAT protein expression levels were determined by dividing absorbance measurements from plates to which T<sub>3</sub> had been added from plates to which no T<sub>3</sub> had been added; plates in the same set were transfected with the same plasmids, their treatment only differed in T<sub>3</sub> addition. The error bars represent Standard Error of the Means. n = 4 replicates.

Effect of Proteasome Inhibition on TR-Mediated CAT Reporter Gene Expression

The interplay between the ubiquitin-proteasome degradation pathway and transcriptional activity of nuclear receptors is an area of ongoing research (Kinyamu et al., 2005; Ferdous et al., 2001; Ferdous et al., 2002; Gillette et al., 2004). Proteasomes in both the nucleus and cytoplasm are capable of liganded or unliganded nuclear receptor destruction; however, it is unclear whether TR ubiquitination and destruction occurs preferentially in a particular cellular compartment. Determining the extent to which the ubiquitination-proteasome pathway exerts control over TR availability is an important
aspect to consider in examining TR-mediated gene expression. As T₃ accelerates proteasomal degradation of TR (Bondzi et al., 2011), CAT ELISAs were performed on cells treated with the proteasome inhibitor, MG132, and T₃ to assess the effects that different treatment protocols involving the proteasome inhibitor and the hormone would have on TR transcriptional activity. All seeded plates were initially transfected with 3.33 µg pGFP-TRα1 and 3.33 µg tk-TREp-CAT and CAT protein levels quantified by CAT ELISA; however, their treatment during the second media change varied. There were three control plates. The first control plate was not treated with T₃ or MG132, the second was treated with 100 nM MG132, and the third was treated with 100 nM T₃. The experimental plate was treated with both 100 nM T₃ and 100 nM MG132. The plates were incubated with these treatments for approximately 12 hours before being lysed during the CAT ELISA. Results from 5 replicates and 9 trials were inconclusive. The experimental plates had higher CAT protein levels relative to the third control in only three trials. This would seem to indicate that proteasome inhibition has a negative impact on TR availability, however, there was a large amount of variability between trials and any differences were not significant (P = 0.667) (Figure 4).
Figure 4. The Effect of Proteasome Inhibition on CAT Reporter Gene Expression: The first bar represents relative CAT Protein Expression in control cells devoid of exogenous T₃ or MG132. The second bar represents relative CAT protein expression in control cells to which MG132 but no T₃ had been added. The third bar represents relative CAT protein expression in control cells to which T₃ but no MG132 had been added. The fourth experimental bar represents relative CAT protein expression in experimental cells to which both T₃ and MG132 had been added. Relative CAT protein expression levels were determined by dividing absorbance measurements from each plate by absorbance measurements from the first control plate. The error bars represent Standard Error of the Means. n = 5 replicates.

Effect of Overexpressing Ubiquitin on TR-Mediated CAT Reporter Gene Expression

Preliminary results from proteasome inhibition assays and immunoprecipitation and western blot data suggest that liganded and ubiquitinated T₃ is bound to chromatin (Subramanian, 2012). These pilot studies led to the question of whether overexpressing ubiquitin would have an impact on CAT expression levels, especially since T₃ presence has documented negative effects on TR half-life (Subramanian, 2012). To investigate this question a similar experimental design was used to the Proteasome Inhibition Assay.
experimental design; there were four plates used in each trial: three control and one experimental. All of the plates were transfected with 3.33 µg pGFP-TRα1; however, the second control and the experimental plate were also transected with 3.33 µg pHA-Ubiquitin. The first and second control did not receive additional T₃ while the third control and experimental plates were incubated for approximately 12 hours with 100 nM T₃.

As with XPO7 and proteasome inhibition CAT ELISAs, ubiquitination CAT ELISAs also had a high degree of variability and any differences were not significant (P = 0.901) (Figure 5). Out of seven replicates and 13 trials, six trials had higher quantified levels of CAT expression in the third control, treated with T₃ but no pHA-Ubiquitin, and seven trials had higher expression in the experimental cells, treated with T₃ and pHA-Ubiquitin.
Figure 5. The Effect of Ubiquitination on CAT Reporter Gene Expression: The first bar represents relative CAT protein expression in control cells that had not been transiently transfected with pHA-Ubiquitin and lacked exogenous T3. The second bar represents relative CAT protein expression in control cells transiently transfected with pHA-Ubiquitin to which T3 had been added. The third bar represents relative CAT protein expression in control cells transiently transfected with pHA-Ubiquitin to which T3 had been added. The fourth bar represents relative CAT protein expression in experimental cells transiently transfected with pHA-Ubiquitin to which both T3 had been added. Relative CAT protein expression levels were determined by dividing absorbance measurements from each plate by absorbance measurements from the first control plate. The error bars represent Standard Error of the Means. n = 7 replicates.
Discussion

XPO7 Facilitates TR Cytosolic Localization Shift and Augments TR-mediated Gene Expression

Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that TRα1 shuttling dynamics were significantly slower with XPO7 knockdown (Subramanian et al., 2015). These prior findings combined with the observation that leptomycin B, a specific CRM1 inhibitor, is insufficient for complete inhibition of TR nuclear export (Bunn et al., 2001), led to further assays in this thesis research to assess the viability of XPO7 in providing a CRM1-independent nuclear export pathway for TR. Here, localization and fluorescence microscopy assays showed that TR had a significantly higher cytoplasmic and whole cell presence in cells overexpressing XPO7, compared to control cells. This data provides evidence indicating a role for XPO7 in facilitating TR nuclear export.

Results from the localization and fluorescence microscopy assays led to the hypothesis that TR-mediated CAT reporter gene expression would decrease in cells overexpressing XPO7, as a smaller nuclear TR presence would seemingly lead to reduced capacity to complex with T3 and bind to TREs to activate gene expression. However, results from transcriptional assays were variable: there was no significant difference between TRα-mediated CAT expression in the presence or absence of XPO7, but TRβ-mediated CAT expression was significantly increased in the presence of XPO7. The disparity in the consistency of the expression patterns between TRα and TRβ may be related to the use of different TREs for promoters of T3 target genes, which influences TR transcriptional activity (McKenna et al., 1999).
The contrasting localization and transcriptional activity data for TRα1, taken in consideration with the generality of XPO7 cargoes, suggests that XPO7 may be facilitating TR nuclear export while simultaneously facilitating nuclear export for corepressors of T3-responsive genes. If the amount of TR left in the nucleus were sufficient to enable transcription from TREs, the effect of corepressor removal may be more stimulatory to transcription than the inhibitory effect of TR export. This hypothesis seems particularly attractive in the context of TRβ, which displayed significant increases in CAT expression despite the cytoplasmic shift observed in cells overexpressing XPO7. Conservation of basic residues in the corepressor SMRT and its interaction partner in a repression domain, GPS2 provide support for the hypothesis that XPO7, seemingly dependent on basic residue recognition, may be facilitating corepressor nuclear export (Oberoi et al., 2011), creating a transcriptional setting conducive to stimulation of TRβ-mediated gene expression. Cytosolic localization and activation of the XPO7 cargo LKB1, increased by T3 in muscle, has been shown to enhance expression of TR coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which stimulates TR-mediated gene expression. The corepressor SMRT undergoes a cytoplasmic shift in response to cellular activity (Guo et al., 2013). A plausible proposal for the cytosolic localization of TR in XPO7 overexpression and the simultaneous increase in TRβ-mediated gene expression may center on nuclear export of LKB1 augmenting TR coactivator and inhibiting corepressor expression.
The Impact of Proteasome Inhibition and Ubiquitin Overexpression on Transcription

The overlap of transcriptional regulation and degradation by the Ubiquitin-Proteasome Pathway (UPP) in many facets of both processes complicates the determination of how experimental manipulation affects TR-mediated gene expression. CAT ELISA results suggest that neither proteasomal inhibition nor ubiquitin overexpression had a significant impact on CAT reporter gene expression under control of a TRE; however, this conclusion may be overly simplistic as these manipulations may activate contradictory mechanisms of transcriptional regulation. For instance, proteasome protease inhibitors increase the amount of ubiquitinated and non-ubiquitinated TRβ1 present while decreasing T3 dependent transcriptional activation (Dace et al., 1999). Similarly, proteasome inhibition increases cellular concentrations of RXR with no concurrent increase in transcriptional activation (Osburn et al., 2001). Proteasome inhibition likely increases the nuclear presence of TR; however; this increased receptor presence does not correlate to increased expression of a TRE-mediated reporter gene, possibly due to a requirement for repressor degradation by functional proteasomes for transactivation, or an accumulation of ubiquitin on TR, or coactivators interfering with their transcriptional activity.

The complexity of these interactions is highlighted in the literature. For example, the transcriptional activity of the yeast transcription factor MET4 is directly inhibited by ubiquitination (Kaiser et al., 2000). In addition, ubiquitin overexpression may enhance pre-existing negative cross-talk interactions between receptors. Estradiol leads to estrogen receptor orchestration of a reduction in intracellular GR levels by increasing
expression levels of the E3 ubiquitin ligase, Mdm2 (Sengupta and Wasylyk, 2001). It wouldn’t be unreasonable to propose similar interactions between TR and other NRs, considering the extensive involvement of TR in multiple signal cascades. Similarly, histone modification cross-talk could also impact transcriptional activity of target genes under control of a TRE, as ubiquitination of histone H2B regulates histone H3 methylation and gene silencing in yeast (Sun and Allis, 2002). Additionally, activation of the SAGA regulated genes GAL1, SUG2, and PHO5 is at least partially dependent on H2B-K123 ubiquitination (Henry et al. 2003), with activation of GAL1 gene being accompanied by dynamic changes in histone H2B ubiquitination levels.

Compounding the difficulty of ascertaining clear relationships between the UPP and the transcriptional entourage of TR target genes are the more well-characterized impacts of the UPP on transcription: promotion of degradation of negative regulators of gene transcription, degradation and recycling of positive factors, disruption of the preinitiation complex enabling transcriptional elongation, and degradation of the elongated form of RNA polymerase II allowing an indefinite number of transcription reinitiations (Yan et al., 2003). A multitude of these influences could be affecting TR-mediated gene expression leading to a complex regulatory picture which cannot be properly detected or assessed with CAT ELISAs which may be insensitive to subtle but significant changes in gene expression.

**Conclusions**

The data presented in this thesis illustrate the intricacy of TR shuttling dynamics and transcriptional activity. In addition to the general CRM1 nuclear export pathway and exportin 5-mediated nuclear export, TR also may use XPO7 as an exportin. The
generality of XPO7 substrates hints that TR nuclear export may only be a small part of
larger signal transduction cascades which may result in promotion of TR coactivator
activity and inhibition of corepressor activity. Additional research is necessary to
determine whether the UPP affects TR-mediated expression patterns and to what extent,
although the potential for UPP influence seems vast in the context of its known
transcriptional impacts. The involvement of TR in a number of physiological processes
like embryonic development, cellular differentiation, metabolism, and cell growth is
directly related to its nucleocytoplasmic shuttling capacity and regulation of receptor
half-life by the UPP. As TRs can directly regulate transcription by binding to TREs of
target genes, nucleocytoplasmic shuttling by the receptor serves as a method for
stimulating or repressing target gene expression. The specificity of response to signaling
cascades is enhanced by regulation of activities of several transcription factors through
nucleocytoplasmic shuttling. Many NRs shuttle between the nucleus and cytoplasm
which affects patterns of target gene expression; NR recycling by the UPP has emerged
as another important regulatory device. Studies have shown that TRβ1 transcriptional
activity is inversely correlated to receptor protein levels suggesting that proteasome-
mediated degradation plays a critical role in the transcriptional activation of TR.
Furthermore, a mutated form of TRβ1 which does not have a decreased half-life in the
presence of T3 as wild-type TRβ1 does, has been implicated in some RTH cases (Dace et
al., 2000).

**Future Directions**

The low sensitivity of CAT ELISAs means that possible UPP impacts on T3-
mediated gene expression may have been obscured in the experiments performed over the
course of this thesis research. Luciferase assays are a more sensitive method for measuring transcriptional output (Adolph, 1996) and could be used to better ascertain the effects of proteasomal inhibition and ubiquitin overexpression on TR transcriptional activity. Should these assays demonstrate a significant UPP impact on TR transcriptional activity, co-immunoprecipitation and western blotting may better demonstrate which specific components of the UPP are interacting with TR and perhaps direct NR cross-talk interactions between TR and other NRs. Furthermore, pull-down or yeast two-hybrid assays could reveal whether or not the interaction between XPO7 and TR is direct and which NES or NESs in TR interact with XPO7, though mutagenesis studies could also be used to this end. Chromatin immunoprecipitation may demonstrate histone modification and histone cross-talk influencing chromatin compaction in promoters for T3–responsive genes. Though X-ray crystallography is outside the purview of this lab, resolution of XPO7 structure alone and complexed with TR would be immensely helpful in elucidating the details of the TR-XPO7 interaction.
References

Introduction:
Overview:

Liu, Y.Y., Brent, G.A. (2010) Thyroid hormone crosstalk with nuclear receptor signaling in metabolic regulation. Trends Endocrinol Metab. 21:166–173


Nuclear Receptors:


Thyroid Hormone:
Bianco AC. Minireview: cracking the metabolic code for thyroid hormone signaling. Endocrinology. 2011;152(9):3306–3311.


Thyroid Hormone Receptor:
Chan, I.H., Privalsky, M.L. (2009) Isoform-Specific Transcriptional Activity of Overlapping Target Genes that Respond to Thyroid Hormone Receptors α1 and β1. Molecular Endocrinology. 23(11):1758-1775


Localization and Shuttling:


Exportin 7:


Ubiquitination-Proteasome Pathway (UPP) and Transcriptional Regulation:


Link to Disease:


Results:


Subramanian, K.S., 2012, Nuclear Export Dynamics of Thyroid Hormone Receptors, College of William and Mary. Williamsburg.


Discussion:

XPO7 Facilitates TR Cytosolic Localization Shift and Augments TR-mediated Gene Expression:


Future Directions: