2012

Nuclear Export Dynamics of Thyroid Hormone Receptors

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A Thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

Department of Biology

The College of William and Mary
August, 2012

APPROVAL PAGE

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

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Approved by the Committee, June, 2012

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COMPLIANCE PAGE

Research approved by

Institutional Biohazard Committee

Protocol number(s): IBC-2010-12-21-7062-laalli

IBC-2011-01-31-7062-laalli

Date(s) of approval: 12-21-2010

01-31-2011

01-31-2012

ABSTRACT PAGE

Thyroid hormone receptors (TRα1 and TRβ1) are nuclear receptors that bind to thyroid hormone to activate or repress target genes involved in metabolism, growth, and development. Although primarily found in the nucleus, TRα1 and TRβ1 rapidly shuttle in and out of the nucleus through nuclear pore complexes. Previously, we showed that TRs exit the nucleus through two pathways, termed CRM1-dependent and CRM1-independent, based on the finding that a potent inhibitor of the export factor CRM1 does not fully disrupt TR shuttling. To investigate which exportins are involved in the CRM1-independent pathway, RNA interference was used to knockdown expression of several different export factors while the effect of knockdown on the nucleocytoplasmic distribution of GFP-TRα1 was assessed in live HeLa cells using fluorescence recovery after photobleaching (FRAP). Knockdown of exportin 5 and exportin 7, alone and together, altered TR's nuclear export dynamics; recovery was markedly slower in photobleached nuclei, indicating that nuclear export was inhibited. To examine whether increased nuclear export affects TR-mediated gene expression, we co-expressed TRα1 or TRβ1, exportin 5, and a thyroid hormone response element (TRE)-mediated CAT reporter gene. CAT ELISA showed a decrease of TRE-mediated CAT reporter gene expression when increased amounts of exportin 5 were present. Furthermore, we showed that TR subcellular distribution shifts to more a cytoplasmic localization when exportin 5 is over-expressed. The physiological significance of TR shuttling remains unclear; thus, we sought to whether post-translational modification of TR by ubiquitination impacts nuclear export. Utilizing immunoprecipitation and western blot analysis, we showed that ubiquitinated TR was found primarily in the cytoplasm in the absence T_3 and bound to chromatin when T_3 was present. Thus, our data suggest that TR ubiquitination plays a role in T_3-mediated gene expression instead of signaling TR for nuclear export. Taken together, our data provide evidence that TR nuclear export is mediated, in part, by exportin 5 and exportin 7, and that disrupting the fine balance between nuclear import and export can lead to changes in TR-mediated gene expression. Our findings have general implications for the regulation of nuclear export of other members of the nuclear receptor superfamily where the misregulation of export may be involved in causing cancer or endocrine disease.

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ACKNOWLEDGEMENTS

I would like to give the warmest thanks to Dr. Allison for all of her support, guidance, and mentorship during my time in the M.S. Biology Graduate Program at the College of William and Mary. I would also like to thank Dr. Bradley and Dr. Kerscher for their guidance, suggestions, and criticism during the writing of this thesis. Furthermore, I would like to acknowledge members of the Allison lab, especially Vinny Roggero and Hallie Nelson. Finally, I would like to thank my family and friends for all their love and support.

Funding for this research was provided by the National Science Foundation.

NUCLEAR EXPORT DYNAMICS OF THYROID HORMONE RECEPTORS

CHAPTER 1: GENERAL INTRODUCTION

Thyroid hormone receptors (TR) are part of the nuclear receptor (NR) superfamily of ligand-activated transcription factors. Responsible for mediating genes involved in metabolism, growth, and development, TRs undergo nucleocytoplasmic shuttling. While NRs shuttle in and out of the nucleus through nuclear pore complexes, they follow distinct nuclear import and export pathways mediated by karyopherins, called importins and exportins, which may be either similar or different among NRs in the superfamily. In particular, NRs can follow both CRM1-independent and CRM1-dependent nuclear export pathways. While TRα1 has been shown to use a cooperative CRM1/calreticulin-mediated export pathway, the CRM1-independent nuclear export pathway remains elusive (Grespin et al., 2008; Mavinakere et al., submitted). Calreticulin is a calcium-binding protein involved in mediating nuclear export of some NRs (Holaska et al., 2002; Prufer and Barsony, 2002); in addition, other macromolecules or signals may mediate NR nuclear import and export. Previously, post-translational modification by phosphorylation has been shown to signal nuclear export of some NRs (Shank et al., 2008). While NRs are post-translationally modified with ubiquitin to mediate protein degradation and transcription, the impact of NR ubiquitination and degradation on nucleocytoplasmic shuttling has not been well studied (Dace et al., 2000). Gene regulation can occur at various levels including protein shuttling, modification, and degradation. Since TR controls genes important for normal cellular function, understanding TR regulation with respect

to T_3-mediated gene expression will help give insight into endocrine disease and cancer.

NUCLEAR RECEPTORS

As integral signals of the endocrine system, hormones are circulated through the bloodstream to target cells where they bind to their respective intracellular nuclear receptors (NRs) or to plasma membrane receptors. With currently 49 identified, NRs are ligand-dependent transcription factors for steroids or non-steroids and are classified into three major categories known as Type I, Type II, and Type III. The latter one is more commonly known as orphan receptors. Even though these NRs are considered ligand-activated transcription factors, some NRs do not need their ligand to activate transcription.

In their ligand-free state, NRs are seen in three major cellular distributions of primarily nuclear, primarily cytoplasmic, or uniform throughout the cell. The current understanding is that glucocorticoid receptors (GR) and androgen receptors (AR) are found primarily in the cytoplasm and are translocated into the nucleus in the presence of their corresponding ligand (Kumar et al., 2006). Progesterone receptors (PR), estrogen receptors (ER), and thyroid hormone receptors (TR), the subject of this thesis, are all found more commonly in the nucleus, while mineralocorticoid receptors (MR) are distributed throughout the cell (Kumar et al., 2006).

In general, addition of a ligand causes complete nuclear localization of NRs although ligand can also trigger mitochondrial localization of GR, ER, and

TR (Kumar et al., 2006). When unliganded GR and AR are found in the cytoplasm, they are bound to chaperone complexes including heat shock proteins (Hsp70 and Hsp90) and immunophilins (Kumar et al., 2006; Maruvada et al., 2003). When ligand is bound and the NR is in its transcriptionally active form, a punctate pattern within the nucleus is noted to be an important regulatory step marking spatial reorganization of the receptor (Black et al., 2004; Kumar et al., 2006). Even though these receptors share structural homology, not all NRs behave similarly; thus, each NR within this superfamily is examined individually.

THYROID HORMONE

Thyroid hormone is important in regulating genes responsible for metabolism, growth, and development. For example, fibrinogen and other proteins involved in the coagulation factor system are under control of thyroid hormone, where gene expression is upregulated in the presence of hormone (Shih, et al., 2004). Triiodothyronine (T_3) and thyroxine (T_4) are two forms of the hormone that can enter a cell through its plasma membrane by passive diffusion like steroid hormones, by facilitated transport, or by binding sites located in the membrane (Yen, 2001). Although these two forms do not differ structurally other than an additional iodine on T_4, T_3 and T_4 are produced in different tissues at different concentrations. T_4 is primarily produced by the thyroid gland and is the major form of the hormone circulating in the blood system (Waung et al., 2011). In the thyroid gland and target cells, T_4 is converted to T_3 by iodothyronine deiodinases, enzymes that remove iodines. While T_3 is considered the active

hormone controlling the major genomic actions within a cell, both T_3 and T_4 have been shown to facilitate genomic or non-genomic effects via a plasma membrane receptor ($\alpha v\beta 3$) through classical signal transduction cascades (Davis et al., 2008).

T_3 exerts genomic effects by binding to thyroid hormone receptors (TRs) and activating or repressing target genes at thyroid hormone response elements (TREs) (Selmi-Ruby and Rousset, 1996). In the absence of T_3, TR represses transcription at positive TREs and activates transcription at negative TREs. Commonly seen as octamer sequences of TAAGGTCA in direct repeats, palindromes, or inverted palindromes, TREs are located in regulatory regions of DNA and are where TRs can homodimerize or heterodimerize with retinoid X receptor (RXR) to control transcription of T_3-responsive genes (Burgos-Trinidad and Koenig, 1999). In addition, the TR corepressor GRP1 (General Receptor for Phophoinositides-1), a nucleotide exchange factor, can decrease TR complex formation at TREs, thereby altering positive and negative regulation by T_3 (Poirier et al., 2005).

Within a cell, T_3 is also important in inducing a conformational change in TRs. Once the hormone and receptor are bound, a conformational change occurs in the receptor at helix 12 in the ligand binding domain so that a new surface of the receptor is revealed and promotes transcriptional activity (Selmi-Ruby et al., 1998). A corepressor (GRP1, N-CoR, or SMRT) dissociates and a coactivator may bind after T_3 binds TRs (Bassett et al., 2003; Calkhoven and Ab, 1996; Horlein et al., 1995). After the coactivator binds to the C-terminal region,

the complex can promote the transcription of target genes. Other components are also associated with the corepressor or coactivator complexes, such as histone deacetylases or histone acetyltransferases, respectively (Baumann et al., 2001; Eckey et al., 2003). With multiple complexes associated with gene expression and repression, understanding how TR associates with not only the ligand and DNA but also coactivators and corepressors is important in understanding its function.

THYROID HORMONE RECEPTORS

Thyroid hormone receptors (TR) α and β are encoded by two genes, THRA and THRB, located on chromosomes 17q11 and 3q24. These genes encode for various receptor isoforms that are developmentally regulated as well as tissue specific (Jazdzewski et al., 2010; Waung et al., 2011). The TR isoforms TRα1, TRα2, TRα3, TRβ1, TRβ2, and TRβ3 arise from alternative splicing of the α and β gene mRNA transcripts (Selmi-Ruby and Rousset, 1996). While TRα1, TRβ1, TRβ2, and TRβ3 are activated when T_3 binds, TRα2 and TRα3 do not bind thyroid hormone and their functions still remain a mystery (Burgos-Trinidad and Koenig, 1999; Jazdzewski et al., 2010). TRα1 and TRβ1 are the two most abundant and widely-expressed isoforms of the receptor and are the focus of this thesis. While both TRα1 and TRβ1 are expressed in skeletal muscle, other tissues express specific TR isoforms. For example, the bone and heart largely express TRα1 while the liver expresses primarily TRβ1 (Rizzoli et al., 1986; Waung et al., 2011). TRα1 and T_3 have also been shown to be

important in skeletal development, as well as bone maintenance (Waung et al., 2011). Even though TRβ1 is very abundant, TRβ2 is only found in the pituitary, hypothalamus, and developing retina (Selmi-Ruby and Rousset, 1996).

Thyroid Hormone Receptor Domains

TRs are comprised of classical domains that are conserved for both steroid and non-steroid NRs: N-terminal transactivation domain, DNA binding domain, Hinge region, ligand binding domain, and a variable C-terminal region. Between TRα1 and TRβ1, the N-terminal transactivation domain is the most variable; this variability between isoforms is common throughout the NR superfamily. In contrast, the DNA binding and ligand binding domains are highly conserved throughout the NR superfamily (Sun et al., 2007). The other TR isoforms have some similarities and differences in their domains. The N-terminal domain of TRα1 and TRα2 are identical; however, the C-terminal region of TRα2 has 120 residues in place of the 40 residues present in TRα1 (Burgos-Trinidad and Koenig, 1999). In contrast, TRβ1 and TRβ2 differ in their N-terminal region (Selmi-Ruby and Rousset, 1996). Since the N-terminal domain lacks conservation within TR isoforms as well as in other members of the NR superfamily, it may be important for the selective effects of receptor isoforms (Selmi-Ruby et al., 1998).

The N-terminal (A/B) domain contains a region termed AF-1, which has transactivation function (Duma et al., 2006). When examining this domain in GR, it was found to activate genes even without ligand present. The DNA binding (C) domain contains two conserved zinc finger motifs and is also where dimerization

occurs. Various localization signals have also been characterized in this domain. Poorly conserved throughout the NR superfamily, the Hinge (D) domain was first thought to only function to separate the two adjacent conserved domains (Nascimento et al., 2006). Current research supports the importance of this domain in nuclear export as many NRs, such as AR and GR, have NESs present here (Shank, 2008); however, TRα1 and TRβ1 have a NLS in the Hinge domain, not a NES (Mavinakere et al., submitted). The structural feature and folding of the Hinge domain in TR has also been implicated in selective TRE recognition (Nascimento et al., 2006). Another interesting note is that the Hinge domain of PR was found to be important for proteasome-mediated transcription, another novel function for NR domains (Tanner et al., 2004). Containing heptad repeats important in dimerization for TR, RAR, or RXR, the ligand binding (E) domain recognizes and binds hormones and, for GR in particular, can interact with heat shock proteins (Selmi-Ruby et al., 1998). An additional transactivation region termed AF-2 is found in this domain and is noted to be weaker than AF-1 (Duma et al., 2006). NRs have been linked to signal transduction pathways, and MAP kinases have been shown to target both the Hinge and ligand binding domains of TR to promote transcriptional activity and stabilization (Chen et al., 2003). The C-terminal (F) domain is also a variable region within NR isoforms. Overall, each NR domain contains multiple functions, thus, exemplifying the complexity of studying these proteins. Altough this thesis research focuses on TRs, understanding their function has implications for understanding how NRs, in general, function as well.

NUCLEOCYTOPLASMIC SHUTTLING

It was previously thought that TRs were continuously bound to DNA in the nucleus; however, current studies have shown that TRs, and other NRs, shuttle between the nucleus and cytoplasm (Baumann et al., 2001; Bunn et al., 2001; Grespin et al., 2008; Maruvada et al., 2003). This shuttling into and out of the nucleus occurs through the nuclear pore complexes (NPCs) of the nuclear membrane (Pemberton and Paschal, 2005). Spanning the inner and outer nuclear membranes to form diffusion channels, NPCs consist of about 30 different proteins, known as nucleoporins, arranged in an eight-fold symmetry (Elad et al., 2009). Structurally, NPCs have a nuclear basket, a spoke-ring complex, and filamentous structures located in the cytoplasm. The pore spans about 50 nm in diameter and allows passive transport of proteins up to about 40 kDa or facilitated transport of larger molecules. Currently, there are several different models that describe how macromolecules are transported through the NPC; however, one common agreement is that the phenylalanine-glycine repeats (FG repeats) located on the nucleoporins are important in the translocation process. Transport proteins, known as karyopherins, have the ability to dock at these sites to move into the NPC.

Mediating protein shuttling through the pore, importins and exportins are karyopherins that recognize proteins by their nuclear localization signals (NLS) and nuclear export signals (NES), respectively (Wagstaff and Jans, 2009) (Table 1). The classical import and export pathways are Ran-mediated, where the Ran-GTP concentration gradient pushes the directionality of protein shuttling (Hoelz,

Import	Cargo
Importin-α	Adaptar protein with importin-β1
Importin-β1	Basic residue NLS cargo, U snRNA, **Glucocorticoid Receptor**
Karyopherin-β2	histones, hnRNPA1, ribosomal proteins
Importin 4	histones, ribosomal proteins **Vitamin D Receptor**
Importin 5	histones, ribosomal proteins
Importin 7	HIV-1 RTC, ribosomal proteins **Glucocorticoid Receptor**
Importin 8	SRP19, **Glucocorticoid Receptor**
Importin 9	actin, ribosomal proteins
Importin 11	UbcM2, rpL12

Import/Export	Cargo
Transportins (1 and 2)	hnRNP (import), mRNA export factor TAP (in cooperation)

Export	Cargo
CRM1	Leucine rich NES cargo,HIV-1 Rev, U snRNA, **Vitamin D Receptor**
Exportin 4	eIF5A, Sox2
Exportin 5	tRNA, Pre-miRNAs, **Androgen Receptor**
Exportin 6	actin, profilin-actin complexes
Exportin 7	p50RhoGAP, 14-3-3σ

Table 1. Importins and exportins. Importins and exportins are identified with their cargoes. The well-known and well-characterized pathways are importin α/β complex for import and CRM1 for export.

2011; Weis, 1998) (Fig. 1). In the nucleus, Ran-GTP binding to the importin and other components causes the release of the cargo; conversely, Ran-GTP promotes exportin binding to cargo (Hood and Silver, 1999; Pemberton and Paschal, 2005; Sorokin et al., 2007). Ran-GDP enters the nucleus in association with the transport protein NTF2. After export into the cytoplasm, Ran-GTP hydrolysis occurring at the cytoplasmic filaments is essential to cause the dissociation of the exportin, cargo, and other components (Fig. 1). Both import and export are, ultimately, energy-dependent because recycling of importins and exportins requires GTP hydrolysis. Karyopherins that mediate the translocation of cargo between the nucleus and cytoplasm are also recycled between these two compartments in order to facilitate the proper movement of their cargo.

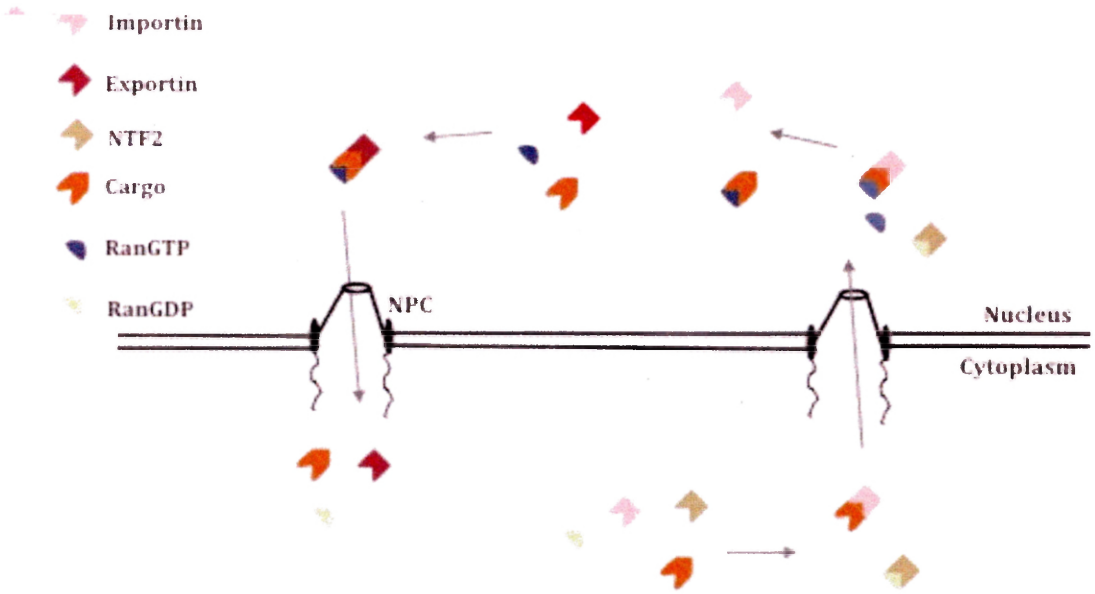

Figure 1. Nuclear import and export. RanGTP and RanGDP concentrations provide directionality for the nuclear import and export pathways. RanGTP promotes nuclear export complex assembly but disassembles nuclear import complexes in the nucleus.

Nuclear Import

A well-characterized nuclear import pathway for a wide array of macromolecules utilizes importin α and importin β heterodimeric complexes (Kumar et al., 2006; Pemberton and Paschal, 2005). Importin α binds to cargo protein containing a NLS with basic residues while importin β mediates interaction of this cargo complex with the NPC (Fig. 2A). Unlike the importin α/β complex, karyopherin β can bind a range of NLSs, such as a sequence with basic residues or an arginine-glycine rich sequence (Pemberton and Paschal, 2005) (Table 1). Structural studies of importins have revealed their flexibility and dynamic shape, and may give insight into their ability to translocate a variety of cargo into the nucleus (Pemberton and Paschal, 2005; Sorokin et al., 2007). Similar to many other cargo, NRs have also been shown to use the classical importin α/β pathway. For example, both AR and GR use the importin α/β complex for nuclear import while GR also uses importin 7 and importin 8 (Nguyen et al., 2009; Pemberton and Paschal, 2005). Recently, we showed that TRα1 follows two nuclear import pathways mediated by the importin α3/β1 complex or by importin 7 (Parente, 2010) (Fig. 2B).

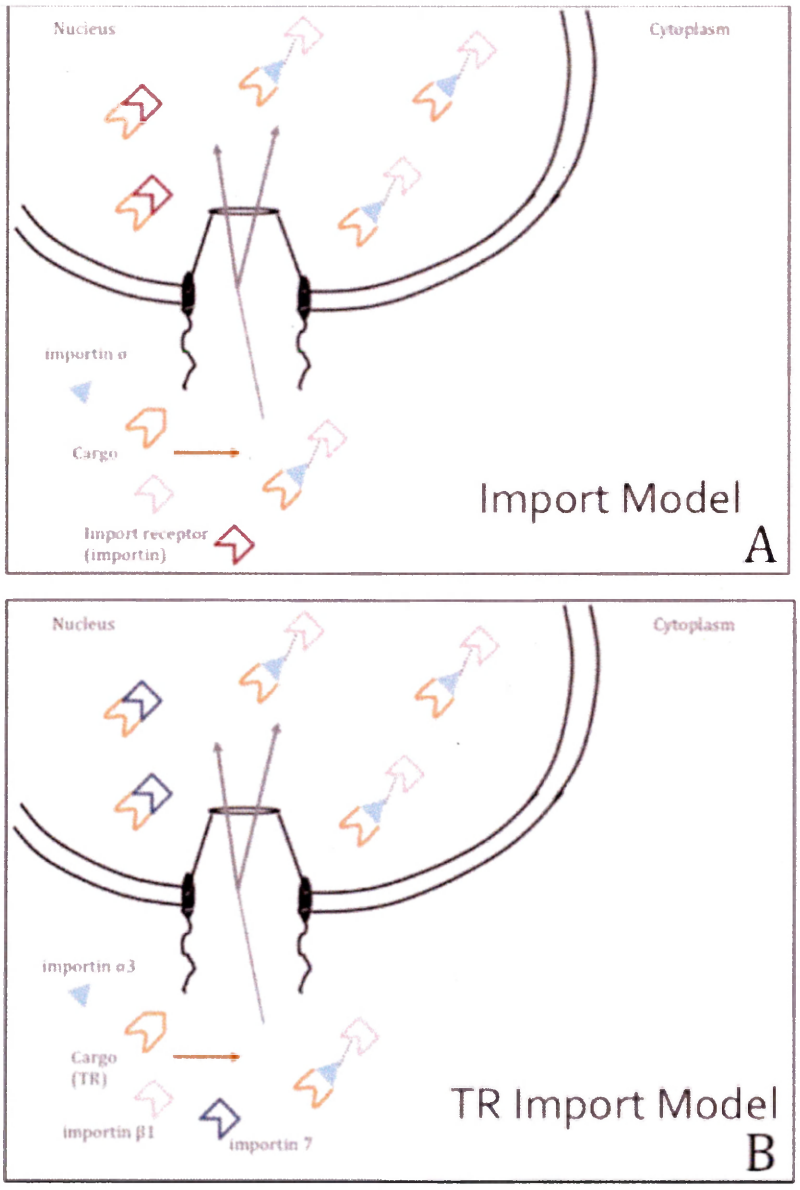

Figure 2. Nuclear import model. A. Classical import model where an import factor, importin, mediates the movement of cargo into the cytoplasm through the nuclear pore complex (NPC). Alternatively, importin α is used as an adaptor protein to mediate import in a complex with importin β1. B. TR import model where import is mediated by importin α3/β1 complex or by importin 7.

Nuclear Export

An exportin that has been characterized and extensively examined is CRM1 (Chromosome Region Maintenance 1), which shuttles a wide variety of cargo containing leucine-rich export signals out of the nucleus (Hutten and Kehlenbach, 2007) (Table 1) (Fig. 3A). While the cargo utilizing this classical CRM1-dependent nuclear export pathway has a leucine-rich NES, some nuclear receptors, such as TR, appear to lack this sequence but continue to use this exportin, in part (Grespin et al., 2008). Steroid nuclear receptors have also been shown to use CRM1-independent nuclear export pathways since their export is insensitive to leptomycin B (LMB), a specific CRM1 inhibitor. Interestingly, the presence or absence of ligand can also change the nuclear export pathway used. For example, VDR export is inhibited by LMB and thus CRM1-dependent only when ligand is not bound (Prufer and Barsony, 2002).

LMB is a well-established reagent commonly used to examine the subcellular localization of macromolecules. It is an antifungal antibiotic with unsaturated, branched fatty acid chains and a δ-lactone ring that was originally shown to inhibit the nuclear export of HIV-1 Rev, which only uses a CRM1-mediated export pathway (Hutten and Kehlenbach, 2007; Kudo et al., 1999). LMB binds CRM1 covalently at a specific cysteine residue (Cys-529), acting as a potent inhibitor of the protein (Kudo et al., 1999). This covalent binding of LMB to CRM1 specifically blocks interaction of CRM1 with a NES.

Other factors have also been implicated in NR export, such as calreticulin (Holaska et al., 2002). Calreticulin is a calcium-binding protein that can be

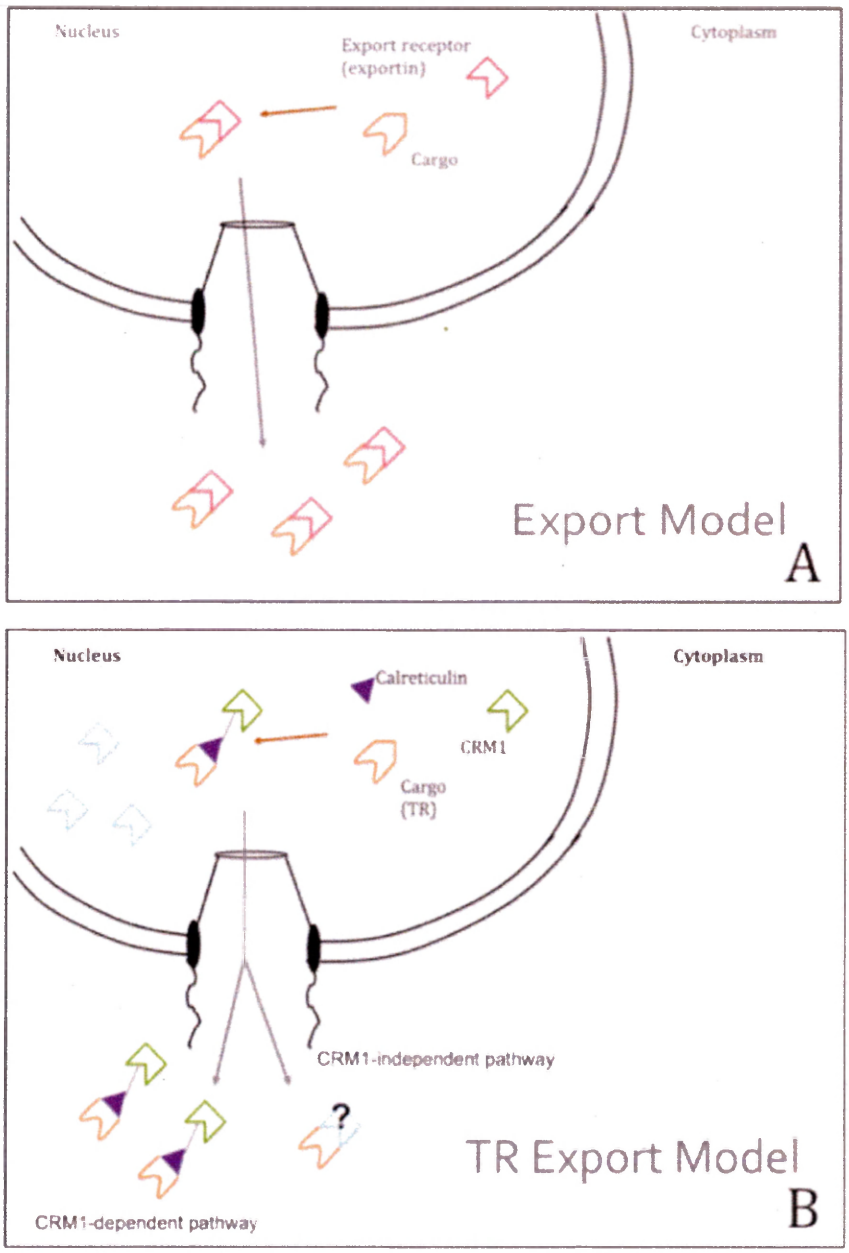

Figure 3. Nuclear export model. A. Classical export model where an export factor, exportin, mediates the movement of cargo into the cytoplasm through the nuclear pore complex (NPC). B. TR export model where two separate pathways may be used. CRM1 and calreticulin interact cooperatively to mediate nuclear export through the CRM1-dependent pathway while a novel, uncharacterized CRM1-independent nuclear export pathway can also be used.

regulated by intracellular levels of calcium ions and is primarily found in the ER lumen (DeFranco, 2001; Holaska et al., 2002). However, many researchers argue against a role of calreticulin in NR export. This is due to the discovery that PEG-induced cell fusion in heterokaryon assays, which are used to study nucleocytoplasmic shuttling, caused cytosolic calreticulin levels to increase above normal levels (Nguyen et al., 2009). While nuclear export of AR does not require calreticulin, TRα1, RXR, VDR, and GR utilize this protein in their nuclear export pathways; GR has been shown to use calreticulin independently of CRM1 with calreticulin binding directly to its DBD (Grespin et al., 2008; (Holaska et al., 2002)Nguyen et al., 2009). Although *in vitro* assays showed that calcium stimulated GR nuclear export, *in vitro* assays with TRα1 showed that increased intracellular calcium levels did not change the subcellular localization of TRα1 (Grespin et al., 2008; Holaska et al., 2002). These findings suggest that calcium is essential for GR export but is not the limiting factor for TRα1 export (Grespin et al., 2008; Holaska et al., 2002).

Recently, TRα1 has been shown to use a cooperative CRM1/calreticulin-mediated export pathway with calreticulin as the adaptor protein essential for CRM1 to export TR (Grespin et al., 2008). While TRα1 does not entirely rely on CRM1 for export, one study suggests that TRβ1 may primarily use a CRM1-mediated export pathway (Maruvada et al., 2003). Other studies emphasize that TRα1 and TRβ1 utilize both CRM1-dependent and CRM1-independent nuclear export pathways, which is a novel finding for NR export (DeLong et al., 2004;

Grespin et al., 2008) (Fig. 3B). This thesis research focuses on identifying the exportins mediating the CRM1-independent nuclear export pathway of TR.

Another exportin has recently been discovered as being important for mediating the CRM1-independent nuclear export of AR. Exportin 5 was found to use a NES located in the DNA binding and Hinge domains of AR (Shank et al., 2008). With this finding, exportin 5 involvement in other NRs and, more importantly, TR nuclear export will be an important focus for this research since NRs have conserved domains and function similarly (Shank et al., 2008).

Nuclear Localization and Nuclear Export Signals

As mentioned previously, macromolecules that move in and out of the NPC using karyopherins have signals that determine their subcellular localization. These signals are part of their amino acid sequence and are termed nuclear localization signals (NLS) and nuclear export signals (NES). By coupling mutagenesis and localization studies, NLS and NES sequences have been found in conserved domains of steroid and non-steroid NRs. Similar to other NRs, TRs have multiple NLS and NES sequences characterized in the Hinge and ligand binding domains (Mavinakere et al., submitted; Nguyen et al., 2009; Sorokin et al., 2007). However, a novel NLS was found in the N-terminal transactivation domain of TRα1 that is not present in TRβ1 (Mavinakere et al., submitted).

Classical bipartite NLS sequences consist of basic residues that commonly use the importin α/β complex or other alternative importins (Sorokin et al., 2007). Depending on the NR, NLS sequences reside in the N-terminal transactivation domain, DNA binding domain, Hinge domain, and even the ligand

binding domain; thus, they are found everywhere. While the classical bipartitie NLS spans the DNA binding and Hinge domains of AR, the NLS for ERα and TR is found in the Hinge domain (Burns et al., 2011). The DNA binding domain of GR and RXR also contains a NLS while GR has an additional NLS characterized in the ligand binding domain (Prufer and Barsony, 2002). A second, less defined NLS has also been found in the ligand binding domain of AR and GR as well as two present in TRs, ligand binding for TRα1 only (Nguyen et al., 2009). Although a NLS is present in the ligand binding domain of TR, this NLS sequence is not fully sufficient for import since a ligand binding domain construct (GFP-GST-GFP-LBD) remains cytosolic (Mavinakere et al., submitted).

Classical NES sequences are leucine rich and utilize a CRM1-mediated export pathway. Many NRs, such as ERα, PR, AR, GR, and MR, have this sequence in their ligand binding domain (Castoria et al., 2009). However, the presence of this leucine rich NES does not gaurantee that the NR will utilize CRM1; for example, AR has this NES sequence but only uses CRM1 when its ligand is absent (Saporita et al., 2003). While this ligand-dependent NES sequence is also found in MR and ER, the NES in AR was found to be both necessary and sufficient for cytoplasmic localization of the receptor (Nguyen et al., 2009; Saporita et al., 2003). As an exception to this rule, TRs have been shown to utilize CRM1 without an identifiable classical NES sequence (Bunn et al., 2001)Grespin et al., 2008).

Not only do the number of localization signals vary in NRs and other proteins but also the signal strength. The NLS and NES strength and

accessibility, therefore, aid in determining nuclear export rate of proteins (Henderson and Eleftheriou, 2000; Kumar et al., 2006). For example, the well-characterized leucine rich NES is greater in strength compared to a novel KNS nuclear export signal (Henderson and Eleftheriou, 2000). KNS was first characterized from the nuclear export of the hnRNP K protein and consists of serines as well as acidic residues. This NES is a CRM1-independent nuclear export signal. Taken together, the NLS and NES strength and accessibility may be an additional regulatory mechanism for many proteins.

Nucleoporins and Nucleocytoplasmic Shuttling

Nucleoporins have also been indicated as binding partners needed to facilitate nucleocytoplasmic shuttling. The Nup214/Nup88 complex consists of Nup214 as the cytoplasmic nucleoporin with FG repeats that binds Nup88. Previously Nup358, another NPC filamentous protein, was the primary nucleoporin thought to bind CRM1; however, current research supports that Nup214 plays the major role in binding of CRM1 to mediate protein export utilizing this pathway (Hutten and Kehlenbach, 2006). Even though Nup358 is implicated in nuclear export, this protein has also been shown to be involved in efficient nuclear import of macromolecules utilizing the importin α/β pathway (Hutten et al., 2007). Nup1 and Nup2 also have high affinities for import complexes, in paricular, importins α and β. Therefore, nucleoporin involvement in nucleocytoplasmic shuttling also aid in mediating the dynamic movement of macromolecules through NPCs.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

Fluorescence recovery after photobleaching (FRAP) studies have been essential to examine intracellular macromolecule movement within the nucleus or between the nucleus and cytoplasm (Deroo et al., 2002; Wang et al., 2004). Whether examining macromolecule movement within one compartment or between two, live cell imaging coupled with FRAP has been a common technique in testing macromolecule movement since the dynamic movement of macromolecules cannot be seen at steady-state.

Previous research studying the rapid shuttling of proteins between the nucleus and cytoplasm used heterokaryon assays where cells of two cell types were fused together in order to examine how the protein moved from one nucleus to another (Baumann et al., 2001; DeLong et al., 2004; Maruvada et al., 2003). This heterokaryon system is no longer beneficial for studying protein translocation since, as noted earlier, it has been shown that PEG-induced cell fusion causes an increase of cytosolic calreticulin released from the ER lumen (Walther et al., 2003). Strong evidence supporting artificial results observed from heterokaryon assays is the shuttling of GR; the heterokaryon system causes GR to shuttle more rapidly compared to what occurs physiologically (Walther et al., 2003).

To examine nuclear export and re-import by FRAP, a single cell with two or more nuclei is used where one nucleus is subject to high laser power in order to irreversibly photobleach a fluorescent protein of interest. Cells with multiple nuclei occur in culture with some frequency as a result of nuclear division without

cytokinesis. The recovery of fluorescence in the bleached nucleus and loss of fluorescence in the unbleached nucleus can then be monitored and analyzed. FRAP has also been used to study movement within the nucleus, when examining transcriptional activity or protein mobility (Deroo et al., 2002; Kino et al., 2004). Both protein movement from the nuclear matrix to chromatin and ligand-induced immobilization within the nucleus have been areas of focus utilizing FRAP (Deroo et al., 2002). This powerful technique formed an integral part of this thesis research.

UBIQUITIN-MEDIATED PROTEASOME PATHWAY

While ubiquitin can mediate protein localization, it is not fully understood how ubiquitin-directed protein degradation mediates protein nuclear import and export. Named for being a ubiquitous protein important in post-translational modification, ubiquitin is attached to proteins in a monomer or poly-chains for protein degradation, localization, stabilization, or transcriptional activation (Chen and Mallampalli, 2009; Jariel-Encontre et al., 2008). Ubiquitin is a highly conserved, 76 amino acid peptide that covalently binds to target proteins as well as other ubiquitin proteins to form chains through an ATP-dependent process (Fig. 4). Three enzymes are involved in this reversible ubiquitination process. First, the E1 ubiquitin-activating enzyme binds the C-terminus of ubiquitin via a thioester bond with a cysteine residue on E1; this is an ATP-dependent step. After activation, ubiquitin is transferred from E1 to the E2 conjugating enzyme by another thioester bond. The last enzyme in the cascade is the E3 ubiquitin ligase

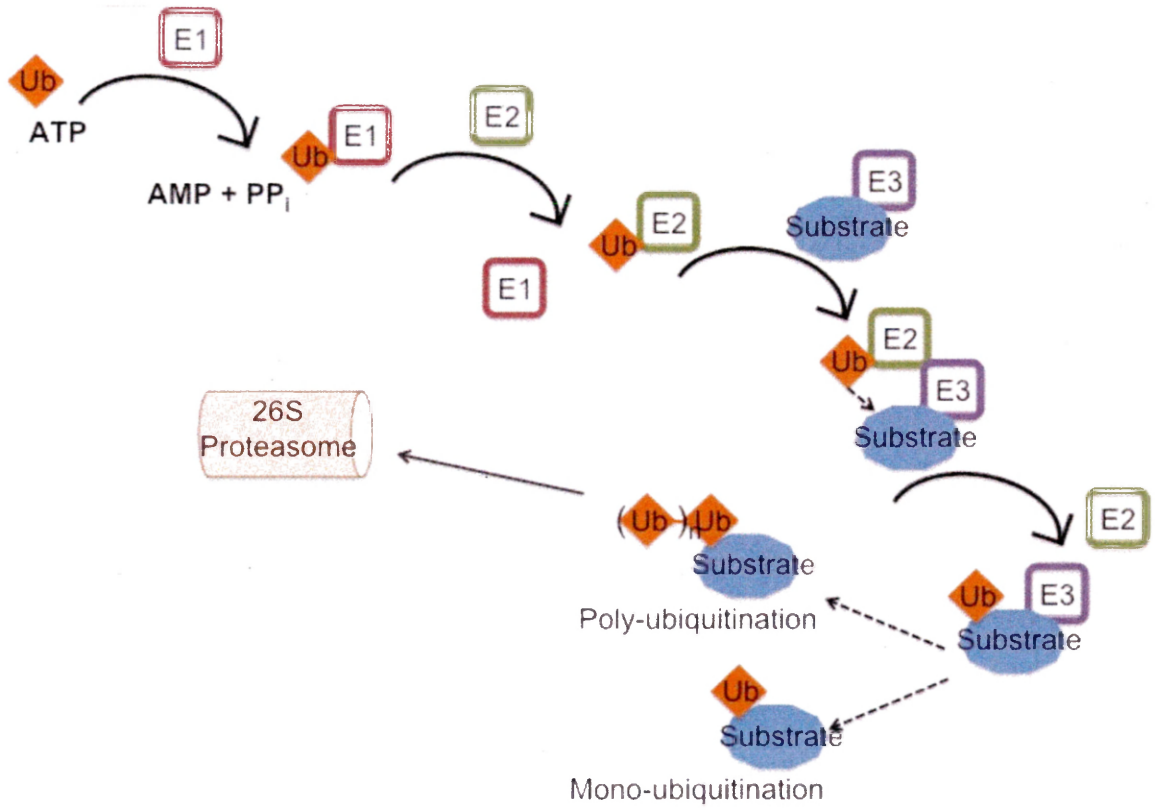

Figure 4. Ubiquitination pathway. Through a series of reactions, ubiquitin is attached to target substrate. E1 ubiquitin-activating enzyme primes ubiquitin through an ATP-dependent step. Next, E1 transfers ubiquitin to an E2 conjugating enzyme. The last step is when the E3 ligase transfers ubiquitin to the target substrate. A mono-ubiquitin chain can lead to changes in subcellular localization or activate or inhibit substrate, while a poly-ubiquitin chain targets substrate for proteasome-mediated degradation.

protein that is important for reaction specificity and binds ubiquitin to the target protein. Not surprisingly, several hundred specific E3 ligases have been characterized to date (Jariel-Encontre et al., 2008). In some cases, however, ubiquitin is attached to target substrates by E2 directly via an isopeptide bond. The isopeptide bond occurs between the C-terminus carboxyl group of ubiquitin and the amino group of the lysine in target substrates. The polyubiquitin chains linked via K29 or K48 can then be recognized by the 26S proteasome for degradation (Faus et al., 2005).

Found in both the cytoplasm and nucleus of eukaryotic cells, proteasomes are vital macromolecular complexes responsible for maintaining protein turnover and degrading over-expressed, misfolded, or non-functional proteins (Jariel-Encontre et al., 2008; Kinyamu et al., 2005; von Mikecz, 2006). The 26S proteasome holoenzyme is composed of two 19S regulatory subunits comprising the lid and base, and one 20S catalytic subunit forming a cylinder-shaped structure (Baugh et al., 2009; Jariel-Encontre et al., 2008; Kinyamu et al., 2005). The 19S cap recognizes ubiquitinated proteins, utilizes ATP to unfold the protein, and then moves the unfolded protein into the proteolytic chamber of the 20S subunit where the protein is cleaved into amino acid residues (Baugh et al., 2009; von Mikecz, 2006).

MG132 is a potent 26S proteasome inhibitor used in determining if proteins utilize the ubiquitin-mediated proteasome degradation pathway (Kinyamu et al., 2005). When MG132 was first used in research, it was considered sufficient to show a laddering with higher molecular weight forms of

the protein by gel electrophoresis to support that it was degraded through this pathway. Researchers now know, however, that proteins can have several different post-translational modifications such as sumoylation, acetylation, and phosphorylation, to name a few. Thus, evidence of direct interactions is needed in order to demonstrate unequivocally that a protein is ubiquitinated. This is commonly shown with immunoprecipitations.

Nuclear Receptor Degradation

In a ligand-dependent or ligand-independent manner, NRs such as ER, AR, GR, and TR are tagged with ubiquitin for degradation through the proteasome degradation pathway (Dennis et al., 2001; Deroo et al., 2002; Nawaz et al., 1999; von Mikecz, 2006; Wang et al., 2009). NRs have been shown to primarily be degraded with the addition of their respective ligand; however, some degradation does occur in the absence of ligand (Nawaz et al., 1999). The mechanism of NR ubiquitination has also been elucidated with the discovery of CHIP (C-terminus of Hsp interacting protein) as the E3 ligase responsible for the last step in NR ubiquitination (Faresse et al., 2010). CHIP has been determined to be important for ER, AR, and MR ubiquitination and may be important in other NR ubiquitination processes since NRs are conserved and use similar mechanisms within the cell (Faresse et al., 2010).

Key players involved in the degradation pathway have been shown to differ among NRs. For example, ERs use two separate degradation pathways depending on the availability of their ligand estrogen (Tateishi et al., 2004). Tateishi et al. made the final conclusion that estrogen is an important molecule

needed to switch between transactivation and degradation of the receptor, which are also under control of two independent E3 ligases. Recently, AR has also been shown to use these same E3 ligases, MdM2 and CHIP, depending on a ligand-dependent phosphorylation event (Chymkowitch et al., 2011). Finally, cytosolic MR, which is the inactive form of the receptor, has been shown to be preferentially polyubiquitinated by CHIP (Faresse et al., 2010). Current research supports that nuclear receptor ubiquitination may occur in two separate subcellular locations as well as via two separate pathways.

A variety of signals, including hormone or coactivator binding, phosphorylation, and acetylation, prepare a protein for degradation (Nawaz and O'Malley, 2004). As more signals for degradation have been revealed, the mechanism of ubiquitination and proteasomal degradation has become more complex. NR phosphorylation occurring in PEST motifs, comprised of amino acid residues Pro, Glu, Ser, and Thr, appears to be the primary signal for ubiquitination, as it may signal recognition by ubiquitin enzymes (Kinyamu et al., 2005). As recognition sites for E2 and E3 enzymes, PEST motifs contain both phosphorylation and ubiquitination sites (Duma et al., 2006; Kinyamu et al., 2005). When a mutation was made in the PEST motif of a GR phosphorylation site, both transcriptional activation and protein degradation were inhibited. Although GR degradation was affected when the PEST motif was mutated, other NRs such as ERα and RXR did not behave similarly with a mutated motif (Brunelle et al., 2011). Degradation of PR has also been shown to be controlled by phosphorylation through a ligand-dependent mechanism (Nawaz et al., 1999).

Coactivator binding has also been shown to be another important signal for NR degradation (Nawaz et al., 1999).

With NR ubiquitination impacted by multiple signals, ligand binding may be the first signal followed by coactivator binding and NR phosphorylation. Interestingly, helix 12 of the NR ligand binding domain appears to be tightly regulated by these signals. The ligand binds to the receptor to induce a conformational change so that coactivators bind directly to this helix 12 region at the C-terminal end (Nawaz and O'Malley, 2004). Helix 12 appears to be important for ligand-dependent degradation of NRs; mutations in helix 12 of ER and RAR, where coactivators are unable to bind, have been shown to prevent this hormone-dependent degradation (Nawaz and O'Malley, 2004). The exact timeline of events and signals for ubiquitination remains unclear and needs more research to understand how each component causes the degradation of NRs.

Thyroid Hormone Receptor Ubiquitination and Degradation

Currently, research is lacking in the molecular mechanism of TR ubiquitination and degradation. T_3 has been shown to decrease the half-life of the receptor from 4.7 to 3.3 hours as well as reducing TR gene expression (Raakat and Samuels, 1981). Although T_3 does not promote TR ubiquitination, T_3 has been shown to promote more rapid degradation of TR through the ubiquitin-proteasome pathway (Dace et al., 2000; Kenessey and Ojamaa, 2005). The ligand binding domain of TRβ1 has also been shown to be essential in the degradation of the protein, which is consistent with other NRs; however, no direct interaction between this domain and ubiquitin has been shown (Dace et al.,

2000; Kenessey and Ojamaa, 2005). Researchers still do not completely understand how ubiquitination affects nucleocytoplasmic shuttling of these proteins, as some make speculations that are not well supported in the literature. Recently, a putative PEST motif was found in TRβ1 (from amino acid residues 211 to 223); however, when the mutation T215A was made, degradation of TR was not impacted (Brunelle et al., 2011). This finding was not similar to other NR PEST motif involvement in ubiquitination and supports that NRs do not always behave similarly. The research from this thesis also focuses on understanding TR degradation and ubiquitination with respect to its impact on nuclear export.

Ubiquitin-mediated Proteasome Pathway and Transcription

The tight coupling of ubiquitin-mediated proteasome degradation and transcriptional activation has been a largely studied area of research. The relationship and control between these two pathways still remains uncertain; however, studies have shown that nuclear receptors bound to DNA are ubiquitinated and that ubiquitin pathway enzymes act as coactivators (Nawaz and O'Malley, 2004). In a ligand-dependent manner, E2 conjugating enzymes, such as UbcH7, and E3 ligases, including E6-associated protein (E6-AP) and RPF1/RSP5, have been shown to bind to nuclear receptors at the DNA response element to promote transcription (Kinyamu et al., 2005; Nawaz et al., 1999). In particular, RPF1 has been shown to enhance ligand-dependent activation of PR and GR while E6-AP enhances ER transcription (Kinyamu et al., 2005). Additional support for the tight coupling of ubiquitination and transcription is the finding that deubiquitination enzymes also act as coactivators. The

deubiquitinating protease USP10 was shown to act as a coactivator for AR ligand-dependent transcriptional activation (Faus et al., 2005). These findings support that the ubiquitin-mediated proteasome pathway is important for NR cycling on DNA response elements and that NR degradation may occur in the nuclear matrix (Nawaz and O'Malley, 2004) .

Interestingly, the E3 ligases involved in NR transcriptional activation are not the same as the previously mentioned E3 ligases that mediate NR ubiquitination (Kinyamu et al., 2005). The E3 ligase MdM2 has been shown to be involved in both NR transcriptional activation as well as degradation; although MdM2 increases ER transactivation, it has been shown to decrease GR transactivation (Kinyamu et al., 2005). Therefore, involvement of ubiquitination in transcriptional activation may also differ for each NR and the mechanism underlying these differences remains to be discovered. The relationship between degradation and transcription also differs between NRs (Deroo et al., 2002). GR and ER have been shown to behave differently in the presence of MG132. With a high concentration of MG132, GR and ER are both seen to have reduced mobility within the nucleus; however, at lower MG132 concentration, GR transactivation increases while ER transactivation decreases (Duma et al., 2006). Proteasome activity has also been shown to be important for AR trancription (Tanner et al., 2004). In particular, the Hinge region of AR plays a role in proteasome-mediated transcriptional activation; however, the exact mechanism still remains unclear (Tanner et al., 2004).

The proteasome itself has also been linked to transcriptional activation (Faus et al., 2005). For example, components of the proteasome complex 19SRP have been shown to play essential roles in TR-mediated transactivation by coordinating the proper loading of liganded TR to a TRE (Satoh et al., 2009). With the discovery of proteasome-mediated transcription, a mechanism has been proposed where the proteasome may disrupt the preinitiation complex to allow elongation to occur for transcription while also aiding in coactivator turnover. This proposed mechanism is supported by the observation that proteasomes accumulate at sites of transcription activity (Faus et al., 2005). Since TRs are transcription factors responsible for regulating genes involved in important cellular processes, understanding the different levels of transcriptional regulation gives insight into the significance of TR shuttling and function, which is a focus of this thesis research.

ENDOCRINE DISEASE AND CANCER

NRs have important roles in maintaining normal cells; alterations in these receptors that interrupt their normal function have been shown to promote tumor proliferation and growth (Cheng, 2003). Receptor mutations may cause reduced or aberrant gene expression, reduced DNA or ligand binding ability, or disrupted shuttling ability (Cheng, 2003; Black et al., 2004; (Bonamy et al., 2005). For example, during prostate cancer disease progression, AR can be imported into the nucleus with transcriptional activation occurring when the ligand is not present; AR behavior is completely opposite of the receptor's normal function

where ligand is needed for both nuclear import and transcription (Pemberton and Paschal, 2005). NR mutations have also been associated with other types of cancers (Bonamy et al., 2005; DeLong et al., 2004; Hsu and Brent, 1998). Breast cancer progression and associated endocrine resistance is another disease where NR mislocalization, ER in particular, was noted (Castoria et al., 2009). TRα1 is also involved in nasopharyngeal carcinoma cellular growth control (Lee et al., 2002). Lee et al. (2002) proposed that TRα1 acts as a tumor suppressor gene in nasopharyngeal carcinoma tumorigenesis. This finding supports the importance of NR regulation for normal cellular function.

As previously mentioned with AR and ER, TR mislocalization may also impact disease progression. For example, v-ErbA is an oncogenic derivative of TRα carried by the avian erythroblastosis virus that causes erythroleukemia and sarcoma in chickens (Bonamy et al., 2005; Bunn et al., 2001). The oncoprotein has also been shown to cause an altered subcellular distribution of TRα and RXR into the cytoplasm (Bonamy et al., 2005). Receptor alterations most commonly lead to dominant negative activities (Burgos-Trinidad and Koenig, 1999; Cheng, 2003).

Mutations in TRβ1 can cause Resistance to Thyroid Hormone (RTH) syndrome. RTH syndrome is an inherited, autosomal dominant endocrine disease where individuals have various mutations found in the TRβ1 gene (Miyoshi et al., 1998). A link between RTH syndrome and misregulation of TRβ1 nuclear export continues to be examined. With more than 70 mutations seen in the TRβ1 gene (Privalsky and Yoh, 2000), it is plausible that the

nucleocytoplasmic shuttling of the receptor is also disrupted. TRβ1 has multiple localization sequences, and any mutation in the NLS or NES would affect the normal physiological shuttling of the receptor as well as impair its function.

THESIS OBJECTIVE

Regulation of TR nuclear export is important in gene expression related to homeostasis. Nuclear export of the receptor itself may also be an important aspect of gene regulation as seen in the dominant negative effects of v-ErbA on TR nuclear distribution. While receptor shuttling has implications in cancer development and other thyroid hormone-related diseases, mutated NRs have been shown to be present in various cancers such as thyroid, kidney, liver, and breast cancer. Finding key players in the nuclear export pathway and understanding the physiological significance for this export will help give further insight into the regulation of T_3-responsive gene transcription, as well as implications for other nuclear export pathways that NRs may follow.

The overall aim of this thesis research was to expand knowledge of the nucleocytoplasmic shuttling pathway and the significance for export of TRs (α1 and β1). Currently, TR has been shown to utilize both a CRM1-dependent and CRM1-independent nuclear export pathway (Grespin et al., 2008; Mavinakere et al., submitted). Multiple NESs have been found in TR; two CRM1-independent NESs were characterized in helix 6 and helix 12 of the ligand binding domain (Mavinakere et al., submitted). The main objective of this thesis research was to determine which exportin(s) mediate the CRM1-independent nuclear export

pathway(s) of TR. Another objective was to examine the physiological significance of TR nuclear export. TR utilizes the ubiquitin-mediated proteasome degradation pathway; however, the compartment in which TR is ubiquitinated and degraded is not known. Does TR have to be exported for degradation? Thus, in particular, the subcellular localization of ubiquitin-mediated proteasome degradation of TR and its impact on nuclear export was examined, as well as the impact of thyroid hormone (T_3 and T_4) availability on nucleocytoplasmic shuttling.

CHAPTER 2: MANUSCRIPT DRAFT FROM THESIS RESEARCH

EXPORTINS 5 AND 7 MEDIATE THE CRM1-INDEPENDENT NUCLEAR EXPORT OF THYROID HORMONE RECEPTORS AND LIGAND REGULATES COMPARTMENT-SPECIFIC UBIQUITINATION

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ABSTRACT

Thyroid hormone receptors (TRα1 and TRβ1) are nuclear receptors that bind to thyroid hormone (T_3) to activate or repress target genes involved in metabolism, growth, and development. Although primarily found in the nucleus, TRα1 and TRβ1 rapidly shuttle in and out of the nucleus through nuclear pore complexes. Previously, we showed that TR nuclear export is not completely blocked when cells are treated with leptomycin B to inhibit the export factor CRM1, suggesting that TR can also exit the nucleus by a CRM1-independent pathway. To determine which exportins are involved in the CRM1-independent pathway, RNA interference was used to knockdown expression of transportin 1, transportin 2, exportin 4, exportin 5, exportin 6, and exportin 7. The effect of knockdown on the nucleocytoplasmic distribution of GFP-tagged TRα1 and TRβ1 was assessed in live HeLa cells using fluorescence recovery after photobleaching (FRAP). Knockdown of exportin 5 and exportin 7, alone and together, altered the nuclear export dynamics of TR; recovery was markedly slower in photobleached nuclei, indicating that nuclear export was inhibited. To determine whether increased nuclear export had an impact on TR-mediated gene expression, we examined CAT reporter gene expression under control of a thyroid hormone response element (TRE) when exportin 5 was overexpressed. CAT ELISA showed a decrease of TRE-mediated CAT reporter gene expression when increased amounts of exportin 5 were present. Further, we showed that when exportin 5 is over-expressed, the distribution of TR shifts to a more cytoplasmic localization. To further examine the physiological significance of TR

nuclear export, we sought to ascertain how TR ubiquitination impacts nuclear export. Our data suggest that ubiquitinated TRα1 does not affect nuclear export of the receptor; ubiquitinated TR was found primarily in the cytoplasm without T_3 and bound to chromatin when T_3 was present. Furthermore, our data provide evidence that TR ubiquitination plays a role in T_3-mediated gene expression, instead of signaling TR for nuclear export. Taken together, our data suggest that TR nuclear export is mediated, in part, by exportin 5 and exportin 7, and that disrupting the fine balance between nuclear import and export can lead to changes in TR-mediated gene expression.

INTRODUCTION

Thyroid hormone (T_3) is important in regulating genes responsible for metabolism, growth, and development. T_3 can enter a cell through the plasma membrane by passive diffusion like steroid hormones, by facilitated transport, or by binding sites located in the membrane (Yen, 2001). Encoded by two genes, THRA and THRB, thyroid hormone receptors (TRα1 and TRβ1) respond to T_3 levels by activating or repressing gene expression. Depending on the target gene, T_3-responsive transcription may be repressed or activated. In the absence of T_3, TR represses transcription at positive thyroid hormone response elements (TREs) and activates transcription at negative TREs.

As a transcription factor primarily found in the nucleus at steady-state, we have previously shown that TRα1 can shuttle between the nucleus and cytoplasm (Baumann et al., 2001; Bunn et al., 2001; Grespin et al., 2008). This

nucleocytoplasmic shuttling occurs through the nuclear pore complexes of the nuclear membrane and is mediated by importins and exportins (Pemberton and Paschal, 2005).

By coupling mutagenesis and localization studies, nuclear localization signal (NLS) and nuclear export signal (NES) sequences have been found in conserved domains of steroid and non-steroid nuclear receptors. Similar to other nuclear receptors, TRs have multiple NLS and NES sequences characterized in the Hinge and ligand binding domains (Mavinakere et al., submitted; Nguyen et al., 2009; Sorokin et al., 2007). However, a novel NLS was found in the N-terminal transactivation domain of TRα1 that is not present in TRβ1 (Mavinakere et al., submitted). Previously, we showed that TRα1 uses a cooperative CRM1/calreticulin-mediated export pathway (Grespin et al., 2008); however, this exact interaction and mechanism remain unclear. Although TRs can follow a CRM1-dependent nuclear export pathway, we know from prior studies that TR nuclear export is not completely blocked when cells are treated with leptomycin B to inhibit the export factor CRM1, suggesting that TRs can also follow CRM1-independent nuclear export pathways. While no CRM1-dependent NES in TRs has been characterized, two NES sequences present in helix 6 and helix 12 of the ligand binding domain utilize CRM1-indpendent export pathways (Mavinakere et al., submitted).

Recently, exportin 5 was shown to be important for mediating the CRM1-independent nuclear export of the androgen receptor (AR); through a NES located in the DNA binding and Hinge domains of AR (Shank et al., 2008). This

came as a surprise because exportin 5 was only thought to be involved in microRNA biogenesis. With this finding, a role for exportin 5 in the nuclear export of other nuclear receptors and, more importantly, TR seemed likely since nuclear receptors have conserved domains and function similarly (Shank et al., 2008).

Despite many studies showing that TR export occurs, the physiological significance of this trafficking remains unclear. Some research points to the possibility that non-genomic effects, such as signal transduction and TR phosphorylation, may promote the nuclear export of TR. Both nuclear export and ubiquitination could be mechanisms for regulating T_3-mediated genes and thus, important in maintaining normal cellular functions. Although T_3 does not promote TR ubiquitination, T_3 has been shown to promote more rapid degradation of TR through the ubiquitin-proteasome pathway (Dace et al., 2000; Kenessey and Ojamaa, 2005). The ligand binding domain of TRβ1 has also been shown to be essential in the degradation of the protein, which is consistent with other nuclear receptors in the superfamily; however, no direct interaction between this domain and ubiquitin has been shown (Dace et al., 2000; Kenessey and Ojamaa, 2005).

Researchers still do not completely understand how ubiquitination affects nucleocytoplasmic shuttling of these proteins. PEST motifs, comprised of amino acid residues Pro, Glu, Ser, and Thr, target proteins for ubiquitination. Recently, a putative PEST motif was found in TRβ1 (from amino acid residues 211 to 223); however, when the mutation T215A was made, degradation of TR was not impacted (Brunelle et al., 2011). This finding differed from other nuclear

receptors, where the PEST motif is involved in ubiquitination, suggesting that nuclear receptors do not always behave similarly. In prior studies, substrate specificity of E3 ligases for nuclear receptors has been shown to be ligand-dependent (Chymkowitch et al., 2011; Tateishi et al., 2004). In particular, estrogen was shown to be important in switching between transactivation function and degradation of the receptor. These processes are under control of two different E3 ligases in the nucleus and cytoplasm, respectively.

To determine which exportins are involved in the CRM1-independent nuclear export pathway of TR, we coupled RNA interference (RNAi) with *in vivo* FRAP experiments and *in vivo* overexpression assays. Our data suggest that exportins 5 and 7 play a major role in mediating the nuclear export of TRα1. We also sought to ascertain whether ubiquitination of TR impacted its nuclear export and where ubiquitinated TR was localized in the cell. Immunoprecipitation assays showed that in the presence of ligand, ubiquitinated TRα1 was primarily nuclear, whereas in the absence of ligand, ubiquitinated TRα1 was found in the cytoplasm. Taken together with *in vivo* FRAP experiments, our data suggest that ubiquitination does not trigger TRα1 nuclear export, instead ligand availability dictates in which compartment ubiquitination of TR occurs.

METHODS

Plasmids - The plasmid pGFP-TRα1 encodes a functional GFP-tagged rat TRα1 fusion protein expressed under human cytomegalovirus promoter control (Bunn et al., 2001). pGFP-TRβ1 encodes a functional GFP-tagged human TRβ1; the

TRβ1 cDNA was cloned into perceiver-M29 vector and was obtained from Capital Biosciences. The pGFP-GST-GFP-TRα1 domain construct expression plasmids were previously described (Mavinakere et al., submitted).

SureSilencing™ shRNA plasmids were obtained from SABiosciences for human transportin 1 (TNPO1, KH19196P), transportin 2 (TNPO2, KH15665P), exportin 4 (XPO4, KH16763P), exportin 5 (XPO5, KH01513P), exportin 6 (XPO6, KP12791P), exportin 7 (XPO7, KP13538P), and a negative control scrambled sequence (Qiagen). Four shRNA expression plasmids were designed per target gene under control of the U1 promoter and containing a puromycin-resistance marker.

pHA-ubiquitin was a gift from K. Fryrear (Eastern Virginia Medical School) and encodes hemaglutinum (HA)-tagged ubiquitin. The pkmyc-exportin 5 was obtained from Addgene (Addgene plasmid 12552) (Brownawell and Macara, 2002). pCAT®3-Basic Vector (E1871) was obtained from Promega, and pCMV-Myc (631604) was obtained from Clontech. tk-TREp-CAT encodes a synthetic TRE linked to a herpes simplex virus thymidine kinase (tk)- chloramphenicol acetyltransferase (CAT) fusion gene reporter construct and was a gift from R. Evans (Salk Institute for Biological Studies).

Cell Culture - HeLa cells were cultured in Minimum Essential Medium (Gibco), supplemented with 10% fetal bovine serum (Invitrogen) and intermittently supplemented with penicillin (100 units/mL)/streptomycin (100 µg/mL), at 37°C under 5% CO_2 and 98% humidity. Cells were grown to 70-90% confluency.

Transient Transfection and Live Cell Imaging - For transient transfections, cells were seeded at 2.0-2.5 x10^5 cells per 3-cm dish (MatTek Corporation). Twenty-four hours after seeding, cells at 70% confluency were transfected with 2 µg plasmid DNA and 4 µL of Lipofectamine 2000 Reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's protocol. Reduced serum medium was replaced with complete medium 9 hours post-transfection.

Twenty-seven hours after transfection, cells were used for microscopy. Prior to imaging, cells were incubated in 2 mL of MEM Alpha medium containing 100 µg/mL cycloheximide (Sigma), penicillin (100 units/mL)/streptomycin (100 µg/mL), 10 µg/mL wheat germ agglutinin conjugated to Alexa Fluor® 350 (Invitrogen), and in some experiments, 2-4 nM LMB (Sigma) or with vehicle (methanol) for 30 min. Cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) and imaged. During the experiment, cells were incubated in MEM Alpha medium containing 50 µg/mL cycloheximide (Sigma), penicillin (50 units/mL)/streptomycin (50 µg/mL), and in some experiments, 2-4 nM LMB (Sigma), 100 nM T_3 or T_4 (Sigma), or with vehicle (methanol).

Images were collected from an inverted Nikon A1Rsi Confocal microscope Ti-E-PFS using a 40X water objective (Nikon). The 488-nm line of krypton-argon laser with a band-pass 525/50 nm emission filter was used for GFP detection while the 405-nm line with a band-pass 450/50 emission filter was used for Alexa Fluor® 350 detection. Images were obtained using the stimulation/bleaching acquisition module of NIS-Elements AR (Nikon).

FRAP was recorded to analyze shuttling of proteins between nuclei within HeLa cells. All FRAP experiments were performed in an OkoLab Incubation System for Ti-E PFS-A1 Confocal System at 37°C under 5% CO_2 and 37% humidity. An initial image was recorded from an area containing a GFP-expressing cell with two or more nuclei using 1-4% laser power from the 488 nm line and using 8-20% laser power from the 405 nm line. One nucleus within the multinucleated cell was exposed at 100% laser power for 10-12 sec using the 488 nm line. After this bleaching exposure, sequential images were taken every 5 min for 24 cycles, 2 hours total. To minimize undesired photobleaching, low laser intensities of 1-4% of the 488 line were again used for post-bleach images. After the 24 cycles, a final image of the cell was recorded from the 488 nm line and the 405 nm line. For quantitative analysis of digitized images, fluorescent intensity values were generated using NIS-Elements AR (Nikon). Bleached and unbleached nuclei were each considered as independent regions of interest. In addition, these values took into account the background brightness levels during each experiment. Intensity values were subsequently normalized so that the total fluorescence within each multinucleated cell after bleaching was equal to 1.

Graphic illustration was used to show the sensitivity of TRα1 nucleocytoplasmic shuttling. Graphs were generated using Microsoft Excel. The mean brightness values for both photobleached and unbleached nuclei were plotted as a function of time post-bleach where the fluorescence intensity was normalized with the overall fluorescence of bleached and unbleached nucei equal to 1.0 (arbitrary units). Similar to the method of analyzing TRα1 shuttling

kinetics previously published, normalized intensity curves for bleached and unbleached nuclei converged towards one another represents the degree of fluorescence equilibration between the two (Grespin et al., 2008). Complete equilibration when bleached and unbleached nuclei are present occurs at 0.5 fluorescent units.

Fixation, Immunofluorescence, and Cell Scoring - HeLa cells were seeded at 2.5-3.0 x10^5 cells per well of a 6-well plate with glass coverslips (Fischer). Twenty-four hours after seeding, cells were transfected with 2 µg plasmid DNA and 4 µL of Lipofectamine 2000 Reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's protocol for 7 to 8 hours. Approximately 24 to 26 hours post-transfection, cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton-X-100. Antibodies used were anti-c-Myc at 1:500 (Clontech) and Cy3-goat anti-mouse at 1:500 (Zymed Laboratories). After processing, coverslips were mounted onto microscope slides in Fluoro-Gel II (Electron Microscopy Sciences) containing the DNA counter stain DAPI (4, 6-diamino-2-phenylindole).

Images were analyzed with an inverted Nikon ECLIPSE TE 2000-E fluorescence microscope (Sigma, Melville, NY). A Nikon Ultraviolet Excitation: UV-2E/C filter block for DAPI visualization, a Blue Excitation: B-2E/C filter block for GFP visualization, and a Red Excitation: T-2E/C filter block for Myc visualization were used with a Nikon Plan Apo 60X objective. Images were collected from an inverted Nikon A1Rsi Confocal microscope Ti-E-PFS using a 40X water objective (Nikon). NIS-Elements AR software (Nikon) were used for

image acquisition and primary image processing. At least 3 replicate transfections were performed per experiment, with a minimum of 100 cells scored per replicate.

RNA extraction, cDNA synthesis, and Real-time PCR - HeLa cells seeded at 6.0-7.0 x10^5 cells per 100-mm dish (Nunc) were transiently transfected with 10 µg shRNA plasmid and 20 µL of Lipofectamine 2000 Reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's protocol. Medium was replaced with complete medium 9 hours post-transfection. Twenty-seven hours after transfection, RNA was extracted using the Aurum™ Total RNA Mini Kit (732-6820) according to the manufacturer's specifications for cultured mammalian cells (BioRad). The DNase I digest incubation was extended to 30 minutes to reduce genomic DNA contamination. To ensure quality RNA samples, total RNA was analyzed using the Agilent 2100 BioAnalyzer. Total RNA samples were run using the RNA 6000 Nano Assay according to the manufacturer's protocol (Agilent Technologies).

600 µg of total RNA was used to synthesize cDNA using the RT^2 First Strand Kit following the manufacturer's specifications (C-03, SABiosciences). First, each RNA sample was added to a separate Genomic DNA Elimination mixture and after, added to a RT cocktail. Quantitative real-time PCR was performed using the Real-Time RT^2 qPCR Primer Assay following the manufacturer's protocol (SABiosciences) with a reaction mix containing RT^2 Real-Time™ SYBR Green/Fluorescein PCR master mix (PA-011), RNase free ddH20, template cDNA, and gene-specific primer. Primers were obtained from

SABiosciences for specific exportins and an internal control, GAPDH, to normalize the raw data. Real-time PCR data were analyzed by the ΔΔCt (Livak) method using the ABI StepOne Software Version 2.1.

Immunoprecipitation - For immunoprecipitation assays, HeLa cells were transfected with 4 μg plasmid DNA and 8 μL of Lipofectamine 2000 Reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's protocol. Reduced serum medium was replaced with complete medium 6 hours post-transfection. Twenty-four hours post-transfection, medium was again changed to complete medium. Medium was replaced 12-16 hours prior to cell lysing with MEM containing 10% charcoal-dextran stripped FBS (Invitrogen) supplemented with or without 100 nM T_3 (Sigma) and with or without 100 nM MG132 (Calbiochem). Cells were rinsed 3 times with D-PBS, scraped, and cytoplasmic, soluble nuclear, and chromatin-bound extracts were prepared using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) according to the manufacturer's instructions. Whole cell extracts were prepared using 1 mL lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM NaF, 10% glycerol, 1% NP-40, 5 mM NEM (Sigma), Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (1 tablet/10 mL).

Extracts were incubated with anti-GFP antibody (Santa Cruz) or anti-HA antibody (Abcam) for 1 hour at 4°C. Extracts and antibody were then incubated with Sepharose Protein G beads (GE Healthcare) rotating for 1.5 to 2 hours at 4°C. The immunoprecipitated material was washed in lysis buffer and eluted in 2X SDS-PAGE sample buffer. Samples were separated by 8% SDS-PAGE.

Replicate Western blots were prepared and probed with anti-Histone 3 (Abcam), anti-HA (Abcam), anti-β-tubulin (Santa Cruz Biotechnology Inc.), and anti-GFP (Santa Cruz Biotechnology Inc.) antibodies, followed by chemiluminescent detection.

Western blotting - The approximate concentration of total protein in whole cell, cytoplasmic, soluble nuclear, and chromatin-bound samples was determined by Nano Drop (ND-1000 Spectrophotometer). For whole cell and cytoplasmic extracts, 40-50 µg of protein were analyzed per lane. Protein amounts to load from soluble nuclear and chromatin-bound extracts were determined by calculating the equivalent cell amounts compared to the cytoplasmic extracts. The samples were separated by 8% SDS-PAGE and transferred to a PVDF membrane using the iBlot Dry Blotting System (Invitrogen). The membranes were incubated overnight in blocking solution (Tris-buffered saline (TBS), 1% bovine serum albumin, 0.1% Tween 20). After 4-6 washes with TBS containing 0.1% Tween 20, the membranes were incubated with primary antibodies for 1.5 hours. All antibodies were incubated separately and used with the following concentrations: anti-Histone 3 at 1:5000 (Abcam), anti-HA at 1:7000 (Abcam), anti-β-tubulin at 1:200 (Santa Cruz Biotechnology Inc.), anti-GFP at 1:200 (Santa Cruz Biotechnology Inc.), or anti-exportin 5 at 1:400 (Abcam). Blots were then washed 4-6 times with TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (GE Healthcare) at 1:25,000 for 1.25 hours in blocking solution. Following the incubation, blots were washed 6-8 times followed by chemilumiscent detection using ECL Plus or Prime

detection reagent (GE Healhcare). Protein size was monitored using the Pre-Stained Kaleidoscope Protein Standards (BioRad).

CAT Enzyme-Linked Immunosorbent Assay (ELISA) - HeLa cells were plated at 6.0 -7.0 x 10^5 cells in 100-mm dishes and transiently transfected for 6-7 hours with 3.33 µg tk-TREp-CAT reporter plasmid, 3.33 µg GFP-TRα1 or GFP-TRβ1 and 3.33 µg pkmyc or 3.33 µg pkmyc-exportin 5. Cells were also transfected with 3.33 µg empty vector (pCAT®3-Basic Vector), 3.33 µg GFP-TRα1, and 3.33 µg pkmyc as a control. Medium was replaced 12-13 hours post-transfection with MEM containing 10% charcoal-dextran stripped FBS (Invitrogen) supplemented with or without 100 nM T_3. After 12 hours, cells were lysed, cell extracts prepared, and extracts were used to determine CAT expression levels by ELISA according to the manufacturer's specifications (Roche Applied Science). Protein concentration was determined by Nano Drop (ND-1000 Spectrophotometer) and adjusted to the same amount of total protein (600 µg). Microplate modules precoated with anti-CAT antibodies were incubated with cell extracts and CAT standards. Digoxygenin (DIG)-tagged secondary antibody to CAT was then bound to the primary antibody-antigen complex. Bound DIG-tagged antibody was quantitated colorimetrically by incubation with peroxidase-conjugated anti-DIG and ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)]) as substrate. Washing of microplate modules was performed after each incubation. The microplate was read at 405 nm against a reagent blank in a microtiter well reader. For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that CAT concentrations of sample extracts fell within

the linear range of the assay. Replicate samples were assayed in each microplate.

RESULTS

TRα1 shuttles more rapidly than TRβ1. Although both TRα1 and TRβ1 have been shown to shuttle between the nucleus and cytoplasm, only the shuttling kinetics of TRα1 have been previously characterized (Grespin et al., 2008). Here, we used FRAP in multinucleated, transfected HeLa cells to compare the kinetics of nuclear export and re-import of GFP-tagged TRα1 and TRβ1.

As a control, transfected HeLa cells were incubated before visualization with fluorescent-tagged wheat germ agglutinin (WGA), a plasma membrane marker; images were taken before and after FRAP experiments to confirm that experiments where no shuttling occurred were conducted in a single cell with multiple nuclei and not nuclei in adjacent, separate cells. All FRAP experiments were also performed in the presence of cycloheximde to ensure that the fluorescence recovery of GFP in bleached nuclei was not due to *de novo* protein synthesis.

Within a cell with two or more nuclei, one nucleus was selected and exposed to high laser power in order to irreversibly photobleach GFP. The unbleached nucleus located in the same cell did not lose fluorescence from the high laser power bleaching step (Fig. 1). Fluorescence intensities for bleached and unbleached nuclei were monitored with a series of images captured every 5 minutes post-bleach.

Consistent with previously reported data (Grespin et al., 2008), TRα1 recovery of fluorescence to bleached nuclei within live transfected cells was 60% (±5%) at 60 min and 75% (±5%) at 120 min (Fig. 1). In contrast, recovery of fluorescence to bleached nuclei for TRβ1 was measured at 43% (±4%) and 59% (±4%) for 60 and 120 min, respectively (Fig. 1). These data are consistent with the finding that TRβ1 lacks a NLS present in the N-terminal transactivation domain of TRα1, which could affect subcellular localization and nucleocytoplasmic shuttling (Mavinakere et al., submitted).

CRM1-independent nuclear export of TRα1 is mediated by exportins 5 and 7. TR is rapidly exported from the nucleus and has been shown to utilize both CRM1-dependent and CRM1-independent mediated nuclear export pathways (Bunn, Grespin et al., 2008; Mavinakere et al., submitted). To characterize the CRM1-independent nuclear export pathway, we coupled *in vivo* approaches using FRAP and RNAi in HeLa cells to examine how knockdown of individual exportins impacts the shuttling kinetics of TR. Using the endogenous microRNA biogenesis pathway, shRNA expression plasmids have been shown to be efficient inducers of RNAi. Here, we used a mix of two shRNA expression plasmids per target gene for efficient knockdown. Real-time qPCR validated that at least 75% knockdown of target exportin mRNA occurred using shRNA expression plasmids (Fig. 2). Since exportin 5 was previously shown to be involved in the nuclear export of the androgen receptor, we predicted that knockdown of exportin 5 would affect the shuttling of TRα1. HeLa cells were co-

transfected with GFP-tagged TRα1 and a panel of shRNA expression plasmids and analyzed by FRAP.

From plotting the fluorescence intensity data, we were able to determine which exportin knockdown affects the dynamic nucleocytoplasmic shuttling of TRα1. Since only exportin 5 has been shown to be involved in NR nuclear export, we did not expect to see an effect from knockdown of transportins 1 and 2 or exportins 4, 6, and 7. In particular, transportins 1 and 2 have primarily been shown to mediate nuclear import of various unrelated macromolecules as well as aiding in mRNA export; therefore, we did not predict that they would be involved in TR nuclear export. As expected, knockdown of transportin 1 and transportin 2 did not show any change in nuclear export and re-import of TRα1, compared to the control where no protein knockdown occurred (Fig. 3). Consistent with previously reported data (Grespin et al., 2008), recovery of fluorescence to bleached nuclei within live cells transfected with a control shRNA was 52% (±4%) at 60 min and 73% (±3%) at 120 min (Fig. 3). Transportin 1 and transportin 2 knockdown had similar shuttling kinetics at 60 and 120 min compared with the control. Fluorescence recovery to bleached nuclei equilibrations were measured at 51% (±6%) and 67% (±5%) for transportin 1 and at 54% (±6%) and 68% (±4%) for transportin 2 (Fig. 3). These data suggest that transportins 1 and 2 are not involved in TRα1 nuclear export or re-import.

We predicted that knockdown of exportins 4, 6, and 7 would not alter TRα1 shuttling kinetics since they have not been shown to be involved in NR export. However, exportin 4 did show a slight disruption in nuclear export and re-

import compared with the control. Fluorescent equilibrations were measured for 60 min and 120 min at 52% (±4%) and 73% (±3%) for the control and 44% (±2%) and 65% (±3%) for exportin 4 (Fig. 4). Further experiments will be required to ascertain if exportin 4 is directly involved in TR nuclear export. As predicted, knockdown of exportin 6 showed similar shuttling kinetics at 60 and 120 min compared to the control, with fluorescent equilibrations to bleached nuclei measured at 49% (±4%) and 71 (±4%) (Fig. 4). These data support that exportins 4 and 6 do not play a role, or at least not an essential role, in the shuttling of TRα1.

Consistent with our hypothesis, exportin 5 knockdown resulted in slower nuclear export and re-import compared to the control; fluorescent equilibration were measured for 60 min and 120 min at 38% (±2%) and 61% (±3%), respectively (Fig. 4). Surprisingly, knockdown of exportin 7 resulted in markedly slower shuttling kinetics of TRα1; fluorescent equilibrations were measured for 60 min and 120 min at 33% (±3%) and 49% (±3%), respectively (Fig. 4). Taken together, these data suggest that exportins 5 and 7 are important for the shuttling of TRα1, suggesting that both these exportins mediate CRM1-independent nuclear export of the receptor.

Since TRs can utilize multiple export pathways, we sought to ascertain whether knockdown of expression of multiple exportins involved in the pathway further inhibit shuttling kinetics. Nuclear export and re-import of GFP-TRα1 was markedly slower when both exportins 5 and 7 were knocked down simultaneouly, compared to when exportin 5 or 7 were knocked down alone. Fluorescence

equilibrations to the bleached nuclei were measured at 30% (±1%) for 60 min and 48% (± 3%) for 120 min (Fig. 5). In comparison, when both exportins 5 and 6 were knocked down, fluorescent recovery in bleached nuclei occurred at 39% (±3%) and 60% (±4%) for 60 and 120 min, respectively. Knockdown of both exportin 5 and 6 did not further inhibit shuttling compared to exportin 5 knockdown alone. Taken together with the previous data, our results provide strong evidence that both exportins 5 and 7 play important roles in the nucleocytoplasmic shuttling of TRα1.

Although knockdown of exportin 5 slowed nuclear export, TRα1 nuclear export and re-import was not completely inhibited. Thus, we wanted to test whether nuclear export could be further inhibited by enhancing knockdown of exportin 5. Since exportin 5 has been shown to be involved in exporting pre-micro RNA and shRNA from the nucleus (Yi et al., 2005), exportin 5 may have a longer half-life, or knockdown of exportin 5 protein levels may need more direct approaches. Previously, it was shown that overexpressing exportin 5 in the presence of shRNA expression plasmids increased the efficiency of RNAi (Yi et al., 2005). Thus, we tested whether, counterintuitively, overexpressing exportin 5 in the presence of exportin 5 shRNA could enhance knockdown. To this end, we analyzed whole cell lysates from HeLa cells transfected with Myc-tagged exportin 5 and exportin 5 shRNA by western blotting with anti-exportin 5 antibody. When knocking down exportin 5, there was only a slight change in exportin 5 protein levels compared to the control shRNA, where no knockdown occurred (Fig. 6A).

In striking contrast, when exportin 5 is overexpressed, there was highly efficient knockdown of exportin 5 protein levels with target shRNA.

After determining that efficient knockdown is seen when transfecting with Myc-Exp5 expression plasmid and shRNA expression plasmid targeting exportin 5, we ran parallel FRAP experiments. In comparison to the control and knockdown of exportin 5 with shRNA alone, we observed a striking reduction in nucleocytoplasmic shuttling of TRα1 (Fig. 6B). The fluorescence equilbrations from unbleached nuclei to bleached nuclei were 27% (±1%) at 60 min and 51% (±4%) at 120 min. In contrast, as shown earlier HeLa cells transfected with a control shRNA expression plasmid showed 52% (±4%) and 73% (±3%) fluorescent equilibration from unbleached nuclei at 60 min and 120 min, respectively (see Fig. 3). These data support the hypothesis that exportin 5-mediated nuclear export is a major pathway followed by TRα1, since the absence of this exportin markedly inhibits nucleocytoplasmic shuttling.

Overexpression of exportin 5 promotes nuclear export of TR. Having shown that nuclear export of TR is inhibited when exportin 5 is knocked down, we sought to test if overexpressing exportin 5 would also alter the subcellular localization of TR. Since knocking down exportin 5 caused a decrease in nuclear export of TRα1, we hypothesized that overexpressing exportin 5 would increase the cytoplasmic localization of TR. To test this prediction, we transiently transfected HeLa cells with GFP-TR (α1 or β1) and Myc-Exp5 (exportin 5) expression plasmids, or Myc expression plasmid as a control, and analyzed TR subcellular distribution. To score cells that were co-transfected with GFP-TR and the control

Myc or Myc-Exp5, HeLa cells were immunostained with anti-Myc antibodies (Fig. 7).

As predicted, TR distribution shifted to a more cytoplasmic localization when exportin 5 was overexpressed ($p<0.001$). Consistent with previous reports (Bunn et al., 2001), 90% (±1%) of cells expressing both GFP-TRα1 and Myc alone had TRα1 localized to the nucleus, while in the remaing 10% (±1%) of cells, TRα1 was present primarily in the nucleus with a small cytoplasmic population (Fig. 7A). In contrast, 52% (±2%) of cells expressing both GFP- TRα1 and Myc-Exp5 had TRα1 localized to the nucleus, while in the remaining 48% (±2%) of cells, TRα1 localized to both the nucleus and cytoplasm. Consistent with published reports that TRβ1 has more of a cytoplasmic population compared to TRα1 (Baumann et al., 2001), 76% (±2%) cells co-transfected with GFP-TRβ1 and Myc expression plasmids had TRβ1 localized primarily to the nucleus with the remaining of cells having 24% (±2%) TRβ1 localized to both the nucleus and cytoplasm (Fig. 7B). Similar to the pattern observed with TRα1, 37% (±2%) of cells expressing GFP-TRβ1 and Myc-Exp5 had TRβ1 localized in the nucleus and 63% (±2%) with TRβ1 localized to the nucleus and cytoplasm. For both TRα1 and TRβ1, the percent of cells with cytoplasmic TR increased when additional exportin 5 was available for nuclear export. Taken together with knockdown experiments, these findings provide additional evidence that exportin 5 plays a key role in TR nuclear export. These data also show that the subcellular localization of TR at steady-state can change dramatically when an exportin involved in its nuclear export pathway is overexpressed.

Enhanced nuclear export of TR decreases T_3-mediated gene expression. To provide additional insight into the physiological significance of nuclear export, we examined the transcriptional activity of GFP-TR (α1 and β1) when overexpressing exportin 5 using a CAT reporter under the control of a positive TRE (Fig. 8). Since TR is a transcription factor needed in the nucleus for T_3-mediated genes, we predicted that overexpressing an exportin involved in nuclear export of TR would cause a decrease in T_3-mediated gene expression. HeLa cells were transiently transfected with GFP-TR (α1 or β1) expression plasmid, Myc or Myc-Exp5 expression plasmid, and tk-TREp-CAT reporter plasmid. Twelve hours after adding 100 nM T_3, CAT ELISAs were performed using whole cell extracts to determine the CAT protein levels. The levels of CAT protein produced were markedly lower in the presence of T_3 when exportin 5 was overexpressed; CAT protein production was 3-fold lower for GFP-TRα1 and 2-fold lower for GFP-TRβ1 compared to the controls. Thus, overexpressing exportin 5 caused a decrease in T_3-responsive gene transcription and directly supports a model in which nuclear export of TRs may be an additional level of control of TR-mediated gene regulation.

Proteasome inhibition disrupts nuclear export of TR. While TRs are degraded through the ubiquitin-mediated proteasome pathway, the subcellular location of TR ubiquitination and how ubiquitination impacts nuclear export is not known. We sought to ascertain whether nuclear export is required for protein turnover. To investigate the relationship between TRα1 mobility and proteasome-mediated degradation, we tested the effect of proteasome inhibition on nuclear export of

TRα1. HeLa cells were transfected with GFP-TRα1 and incubated with the proteasome inhibitor MG132 prior to and during FRAP experiments. MG132 caused a markedly slower nuclear export and re-import of TRα1, compared with shuttling in the absence of MG132 (Fig. 9). Recovery of fluorescence to bleached nuclei in live cells treated with MG132 was measured at 22% (±2%) at 60 min and 42% (±3%) at 120 min. In contrast, in the absence of MG132, recovery to bleached nuclei was measured at 60% (±5%) at 60 min and 75% (±5%) at 120 min (Fig. 9). When the proteasome is blocked, the shuttling of TRα1 was also inhibited, suggesting that an active proteasome is needed for normal TR function, in particular nuclear export.

Ubiquitinated unliganded TRα1 localizes to chromatin and the cytoplasm, while ubiquitinated liganded TRα1 localizes primarily to chromatin. TR is degraded through the ubiquitin-mediated proteasome pathway and is rapidly degraded in the presence of T_3 (Bondzi et al., 2011; Dace et al., 2000). To investigate the localization of ubiquitinated TRα1, we examined cytoplasmic, soluble nuclear, and chromatin-bound fractionations of HeLa cells co-transfected with GFP-TRα1 and HA-Ub (ubiquitin) expression plasmids. Immunoprecipitation and western blot analyses were performed with subcellular fractionations to identify where ubiquitinated TRα1 localizes within the cell. Nuclear Histone H3 and cytoplasmic β-tubulin were also examined to ensure fractionations were clean, and samples with equivalent cell amounts were analyzed. As shown in Figure 10A, addition of MG132 to HeLa cells increased the level of TRα1 protein in the soluble nuclear, chromatin-bound, and whole cell extracts and increased the level of ubiquitinated

TRα1 protein in the cytoplasmic, chromatin-bound, and whole cell extracts. Consistent with previous research (Dace et al., 2000;(Kenessey and Ojamaa, 2005), these data support that TR is degraded through the ubiquitin-mediated proteasome pathway.

To examine how T_3 affected ubiquitinated TR localization, T_3 was added with MG132 prior to fractionations. When the proteasome was inhibited with MG132, ubiquitinated TRα1 was found both in the cytoplasm and bound to chromatin (Fig. 10B). When the proteasome was inhibited and T_3 was present, ubiquitinated TRα1 was primarily bound to chromatin (Fig. 10B). Similar to previous findings, TRα1 was ubiquitinated in the absence and presence of T_3, indicating that both unliganded and liganded TRα1 are degraded through the ubiquitin-mediated proteasome pathway. In contrast, the localization of ubiquitinated TRα1 changed depending on availability of ligand. Since there has been a link made between ubiquitination as well as proteasome-mediated degradation to transcriptional activation, these data also suggest that ubiquitinated liganded TR, may be involved with transcriptional control as it remains bound to the chromatin.

Ubiquitination of the Hinge domain of TRα1 is ligand-dependent, while ubiquitination of the A/B domain, DBD, and LBD do not require ligand. Although the ligand binding domain has been shown to be important in TR degradation as well as other NRs such as ERα (Dace et al., 2000; (Lonard et al., 2000), the ubiquitination sites on TR remain to be elucidated. We first sought to determine which TR domain(s) contained putative ubiquitination sites using UbPred, an

online predictor of potential ubiquitination sites (Fig. 11). TRα1 has four distinct, functional domains, A/B domain, DNA binding domain (DBD), Hinge domain, and ligand binding domain (LBD). The A/B domain, Hinge domain, and LBD all have lysines with high prediction sites for ubiquitination. To investigate which domains are in fact ubiquitinated, an *in vivo* approach was taken with HeLa cells co-transfected with GFP-GST-GFP- domain constructs and HA-Ub in HeLa cells. Twelve to sixteen hours prior to cell lysing, DMSO or MG132 with or without T_3 was added to cells.

First, as a control, we sought to demonstrate that full-length TRα1 and ubiquitin interact (Fig. 12A). Consistent with previous research (Dennis et al., 2001; Kenessey and Ojamaa, 2005), these data show that TRα1 is ubiquitinated. Next, we examined which TRα1 functional domains are ubiquitinated in the presence or absence of ligand. In the presence and absence of T_3, the A/B domain and LBD were both ubiquitinated. The LBD is also supported in the literature to be an important domain for mediating TR degradation (Dace et al., 2000). However, in the absence of T_3, the DBD was ubiquitinated, while in the presence of T_3, the Hinge domain was ubiquitinated (Fig. 12B). For other NRs, the A/B domain and LBD have been shown to be important for NR degradation due to the transactivation functions present in these domains (AF1 and AF2). While previous literature and UbPred do not predict that the DBD can be ubiquitinated, we show that the DBD, in fact, can be ubiquitinated in the absence of T_3. These data provide evidence that all functional domains of TRα1 have ubiquitination sites.

T_4 promotes more rapid shuttling of TRα1. T_3 has been shown to promote more rapid degradation of TR, promote gene expression of T_3-mediated genes (at positive TREs), and cause a more complete nuclear localization of TR. Since ligand is important in genomic and non-genomic functions of TR, we sought to ascertain whether T_3 and T_4 impact the nuclear export of TR. HeLa cells were transfected with GFP-TRα1, and cells were incubated prior to and during FRAP experiments with 100 nM ligand (T_3 or T_4). We predicted that T_3 would inhibit nuclear export since it promotes gene expression, while T_4 may promote export since it has been linked with signal transduction.

In contrast to our prediction, the addition of T_3 did not have a large effect on shuttling of TRα1; if anything, there was only a slight decrease in shuttling (Fig. 13). Recovery of fluorescence to bleached nuclei within live cells was measured at 60 min with 44% (±2%) equilibration and at 120 min with 70% (±2%) equilibration (Fig. 13). These results are similar to FRAP experiments in the absence of T_3, during which TRα1 shuttling kinetics were only slightly more rapid: recovery to the bleached nuclei was measured at 60% (±5%) equilibration at 60 min and 75% (±5%) equilibration at 120 min (Fig. 13).

In striking contrast, T_4 caused a more rapid nucleocytoplasmic shuttling of TRα1, with fluorescence equilibration to bleached nuclei measured at 50% (±2%) and 79% (±3%) for 60 and 120 min, respectively (Fig. 13). Our data show that T_4 causes TRα1 to shuttle more rapidly between the nucleus and cytoplasm, in comparison to not only the control but also to the presence of T_3. These findings suggest that T_4 signals TR for nuclear export and re-import.

DISCUSSION

Previously we showed that TRα1 participates in rapid nucleocytoplasmic shuttling and can follow both CRM1-dependent and CRM1-independent nuclear export pathways (Bunn et al., 2001; Grespin et al., *2008;* Mavinakere et al., submitted). Here, we provide evidence for a novel mechanism for the CRM1-independent nuclear export pathway of TRs. By coupling *in vivo* RNAi and FRAP experiments, we showed that knockdown of exportin 5 and/or exportin 7 gene expression disrupts nuclear export and re-import of TRα1. While knockdown of exportin 5 inhibited shuttling kinetics, overexpression of exportin 5 changed TR subcellular localization to a more cytoplasmic distribution. We showed that when TR localization shifted to a more cytoplasmic distribution, T_3-mediated gene expression drastically decreased. Taken together, our data provide evidence that exportin 5 is important in mediating the nuclear export of TRα1 and aids in regulating T_3-mediated gene expression. From immunoprecipitation and western blot analysis, we also showed that TR is ubiquitinated, and that ubiquitinated TR in the absence of T_3 is found in the cytoplasm but not in the presence of T_3. Our data support a model in which TR can be degraded via two separate ubiquitin-mediated proteasome pathways, and suggest that TRs are ubiquitinated while bound to chromatin.

TR isoforms differ in shuttling kinetics

In prior studies, we showed that TRα1 shuttles rapidly between the nucleus and cytoplasm of HeLa cells and that TRβ1 lacks a NLS found in the N-terminal domain of TRα1 (Grespin et al., 2008; Mavinakere et al., submitted).

Here, we showed that the shuttling kinetics of TRα1 and TRβ1 differ; TRα1 shuttles more rapidly than TRβ1. The lack of the additional NLS in TRβ1, therefore, impacts the shuttling of this receptor. The number and strength of signal sequences (NLSs and NESs) differ in the NR superfamily, which is also supported by our data. The NLS and NES strength and accessibility, therefore, aid in determining the nuclear export rate of proteins (Henderson and Eleftheriou, 2000; Kumar et al., 2006). These data provide support that the number of signal sequences affects the shuttling dynamics of TRs and may also aid in any differing behaviors of each isoform.

Exportins 5 and 7 mediate TR nuclear export

While previous research suggests that NRs follow similar nuclear export pathways, conflicting data also show that NRs have distinct nuclear export behavior. TRα1 was previously shown to utilize calreticulin and CRM1 cooperatively in order to exit the nucleus (Grespin et al., 2008). Other NRs have also been shown to use calreticulin and CRM1 such as the glucocorticoid receptor (GR); however, some NRs use calreticulin or CRM1 independently of each other (Holaska et al., 2002)Nguyen et al., 2009). Recently, AR has been shown to utilize exportin 5 in its CRM1-independent nuclear export pathway through interaction with a NES located in the DNA binding domain (Shank et al., 2008). This is the first exportin other than CRM1 that has been implicated in the mechanism of NR export; thus, we predicted that exportin 5 would be involved in mediating the nuclear export of TR.

RNAi utilizes the endogenous mechanism for microRNA bigenesis where pri-miRNA is processed to pre-miRNA in the nucleus, exported into the cytoplasm through an exportin 5-mediated nuclear export pathway, and further matured in the cytoplasm where silencing of genes at the mRNA level occurs. It is important to note that exportin 5 is utilized in the process of miRNA and shRNA biogenesis, so knocking down this exportin may have widespread effects. In a prior study examining shRNA efficiency, exportin 5 overexpression while utilizing shRNA for RNAi was shown to cause a more potent knockdown (Yi et al., 2005). This approach was taken to further examine exportin 5 knockdown on TR shuttling kinetics. Coupled with overexpression experiments, our data support the hypothesis that TRα1 can utilize an exportin 5-mediated pathway; this finding suggests that exportin 5 may be important for nuclear export of other NRs as well.

Surprisingly, we showed that exportin 7 is also a key player in the shuttling of TRs. Mediating cargo with folded motifs including basic residues, exportin 7 was previously shown to bind diverse cargo with variable NESs including eIF4A1, p50RhoGAP, and14-3-3σ. Exportin 7, also known as RanBP16, has been described as being similar to CRM1 due to its broad range of cargoes (Mingot et al., 2004). With our novel finding, we predict that other NRs in the superfamily also utilize this exportin in their nuclear export pathways.

Unliganded TRα1 can be ubiquitinated in the cytoplasm

Our data shed light on the physiological significance of TR nuclear export. Continued research has been in the area of post-translational modifications of

NRs that may direct their subcellular localization. While NR phosphorylation may change a protein's subcellular localization, phosphorylation has also been shown as a priming step for signaling ubiquitination (Brunelle et al., 2011). Consistent with previous studies, we showed that TR is poly-ubiquitinated and degraded through the proteasome-mediated degradation pathway (Bondzi et al.; Dace et al., 2000; Kenessey and Ojamaa, 2005).

We show here that ligand-dependent ubiquitination does not fully explain the nuclear export dynamics of TRα1. FRAP experiments with GFP-TRα1 showed that T_3 slightly inhibits nuclear export and re-import while immunoprecipitation and western blot analysis showed that T_3 shifted ubiquitinated TR localization to primarily chromatin-bound. Taken together, our data suggest that ubiquitination of TR does not substantially direct nuclear export; other yet to be identified post-translational modifications may be important for mediating nuclear export. Since T_3 has been shown to increase degradation of TR, presumably if ubiquitination was a factor in TR nuclear export, then T_3 should drastically increase nuclear export of TR and increase the cytoplasmic population of ubiquitinated TR. However, when the proteasome was inhibited, TR nuclear export was also impacted. MG132 is commonly used to inhibit proteasome-mediated degradation of proteins (Kinyamu et al., 2005); thus, it has been a widely used reagent in examining protein degradation. Here, TR mobility was drastically impaired and may point to the importance of an active proteasome for normal TR function. Other NRs, such as ER and GR, have impaired mobility and are localized to the nuclear matrix, therefore, also

disrupting nuclear export (Deroo et al., 2002). Our findings point to the possibility that TR redistributes and becomes associated with the nuclear architecture after proteasome inhibition.

Interestingly, the estrogen receptor (ER) has been shown to be ubiquitinated in two separate locations by two separate E3 ligases, dependent on the presence or absence of ligand (Tateishi et al., 2004). When ERα was not bound to estrogen, it was ubiquitinated and bound to CHIP (C-terminus of Hsp interacting protein) in its ligand binding domain. As ER is primarily nuclear; the finding that CHIP is primarily cytoplasmic suggests that ER must localize to the cytoplasm for CHIP-mediated ubiquitination to occur. Taken together with our data, a mechanistic model of TR ubiquitination can be proposed. In fact, we show that unliganded TRα1 can rapidly shuttle between the nucleus and cytoplasm. When unliganded TRα1 is in the cytoplasm, some of the receptor may be ubiquitinated at that location, supported by our finding that cytoplasmic TRα1 was ubiquitinated. We also showed that even though liganded TRα1 could shuttle, the protein was not ubiquitinated in the cytoplasm (Fig. 14). This suggests that liganded TRα1 was potentially ubiquitinated only in the nucleus. Our data also suggest that TRα1 may have multiple ubiquitin-mediated degradation pathways.

Since E3 ligases are the last step important for specificity in the ubiquitination process, how E3 ligases regulate the kinetics of NR shuttling would be a novel finding. E3 ligases have been seen to regulate and affect the localization of proteins. In particular, p53 cellular localization was changed

depending on MdM2 concentrations (von Mikecz, 2006). MdM2 is a ubiquitously localized E3 ligase. Interestingly, when the level of MdM2 was high, then p53 was poly-ubiquitinated in the nucleus and degraded while low levels caused mono-ubiquitination and nuclear export of p53 (von Mikecz, 2006).

TRα1 ubiquitination occurs in a ligand-independent and ligand-dependent manner

As multifaceted proteins, various regions of NRs have been implicated in their ubiquitination and degradation. The transactivating regions found in the N-terminal and ligand binding domains of NRs have been shown to be important in ubiquitination (Duma et al., 2006). While ER is ubiquitinated in both regions, the ligand binding domain of TRβ1 appears to be important for the degradation of the protein (Dace et al., 2000). Our data suggest that all of the domains have potential ubiquitination sites depending on the presence of ligand. The N-terminal and ligand binding domains of TR can be constitutively ubiquitinated, which means they are ubiquitinated no matter whether the ligand is present or not. In contrast, ubiquitination of the DNA binding and Hinge domains occur in a ligand-independent or ligand-dependent manner. While the DNA binding domain has not been implicated in NR ubiquitination, the DNA binding domain of p53 has been shown to have multiple ubiquitination sites (Chan et al., 2006). Another interesting note is that the Hinge domain of PR was found to be important for proteasome-mediated transcription, another novel function for NR domains (Tanner et al., 2004). While our data show that the Hinge domain is ubiquitinated in the presence of ligand, our data point to the possibility that the

Hinge domain of TR may also be involved in proteasome-mediated transcription. A limitation of our findings is that we examined the domains separately and did not look at the full-length protein, which may play a factor in what Lys residues are, in fact, ubiquitinated. Since we only tested the domains, examining the full-length protein with mutagenesis analysis would give more information on what domains may work together or how other physiological signals within the cell may affect ubiquitination.

Conclusion

In summary, the results presented here reveal the complexity of TR shuttling, which uses multiple pathways and provides further evidence for CRM1-independent nuclear export of TR. The mechanistic detail and physiological significance of nuclear export remain to be elucidated for proteins found primarily in the nucleus at steady-state. The challenge of studying these proteins is evident in their localization, since their nuclear export is rapid and transient. Determining the physiological significance of TR nuclear export is an important area of research as T_3-mediated gene expression is highly sensitive to TR localization changes. With the discovery of two CRM1-indpendent NESs in the ligand binding domain of TR (Mavinakere et al., submitted), continued *in vivo* experiments will be needed to determine the exportins that can bind to these NESs and under what cellular conditions. Insight in determining where these exportins bind would not only reveal additional information for the nuclear export mechanism of other members of the NR superfamily but would also aid in determining the NES sequence these exportins bind in other cargoes. The

finding that TRs can use multiple export pathways also deomonstrates that nuclear export is required for normal TR function. As a whole, these data provide insight into the mechanism and physiological significance of TR nuclear export and therefore, T_3-mediated gene expression.

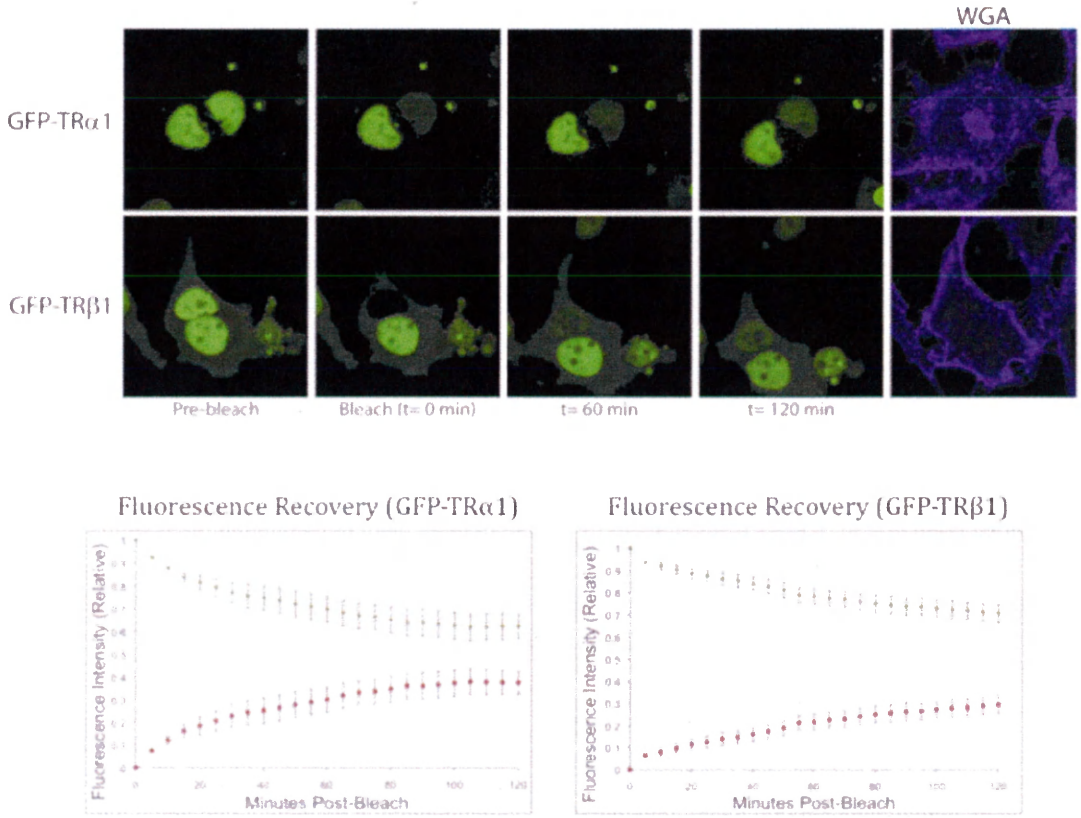


Figure 1. **TRβ1 shuttles markedly slower than TRα1**. HeLa cells were transiently transfected with GFP-TR (α1 or β1) expression plasmid. FRAP experiments were performed in multinucleated live cells to monitor the movement of GFP-TR from unbleached to bleached nuclei (n=4, TRα1; n=4, TRβ1). In order to identify cells with two or more nuclei, HeLa cells were incubated before visualization with wheat germ agglutinin (WGA), a plasma membrane marker, with images taken before and after each experiment. Fluorescence recovery graphs showing the relative movement of GFP-TR in and out of the nuclei were generated. Green diamonds represent relative fluorescence intensity within unbleached nuclei, while red squares represent intensity within bleached nuclei. Error bars indicate SEMs.

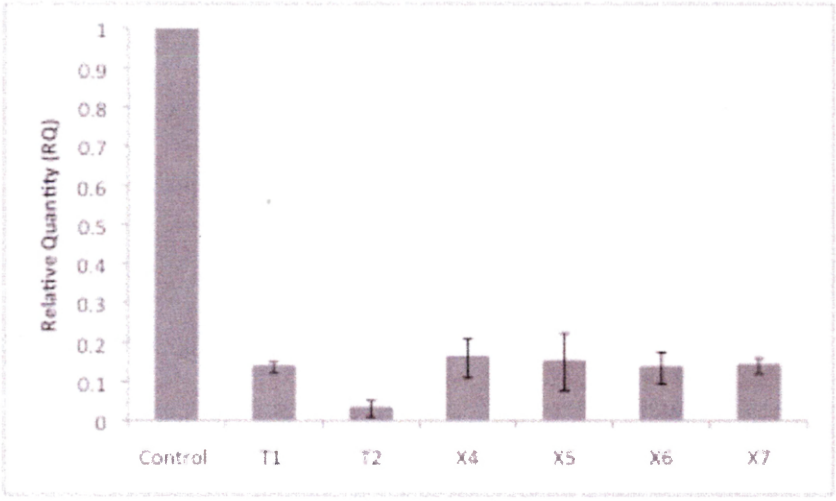


Figure 2. **shRNA knockdown of target gene mRNA**. HeLa cells were transiently transfected with a panel of shRNA expression plasmids. Twenty-seven hours post-transfection, cells were lysed and RNA extracted, cDNA was synthesized, and then real-time PCR was perfomed. The relative quantity (RQ) of target gene mRNA expression was determined by the ΔΔCt (Livak) method. GAPDH was used as an internal control to normalize the raw data. A control shRNA expression plasmid, where no mRNA knockdown occurred, was also used, as indicated on the graph (RQ=1). Knockdown of target gene mRNA was at least 75% with shRNA expression plasmids. Error bars indicate SEMs. (Control, control shRNA; T1, transportin 1; T2, transportin 2; X4, exportin 4; X5, exportin 5; X6, exportin 6; X7, exportin 7)

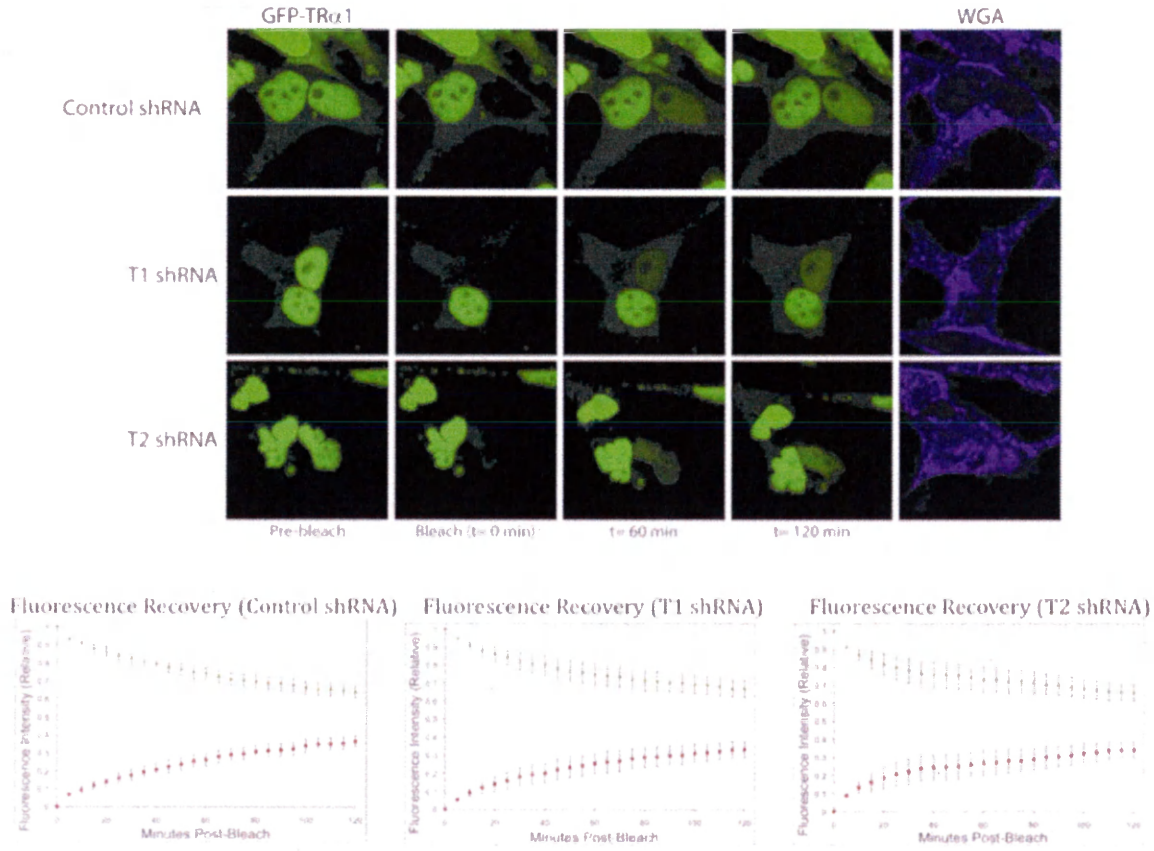


Figure 3. **Knockdown of transportin 1 or transportin 2 does not disrupt shuttling of TRα1.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and shRNA expression plasmid targeting transportin 1 (T1 shRNA) or transportin 2 (T2 shRNA). Parallel experiments were run with a control shRNA expression plasmid (Control shRNA), where no protein knockdown occurs. FRAP experiments were performed in multinucleated live cells to monitor the movement of GFP-TRα1 from the unbleached to the bleached nuclei (n=7, Control shRNA; n=6, T1 shRNA; n=6, T2 shRNA). Fluorescence recovery graphs show the shuttling of GFP-TRα1 with red squares indicating relative intensity within bleached nuclei and green diamonds indicating relative intensity within unbleached nuclei. Error bars indicate SEMs.

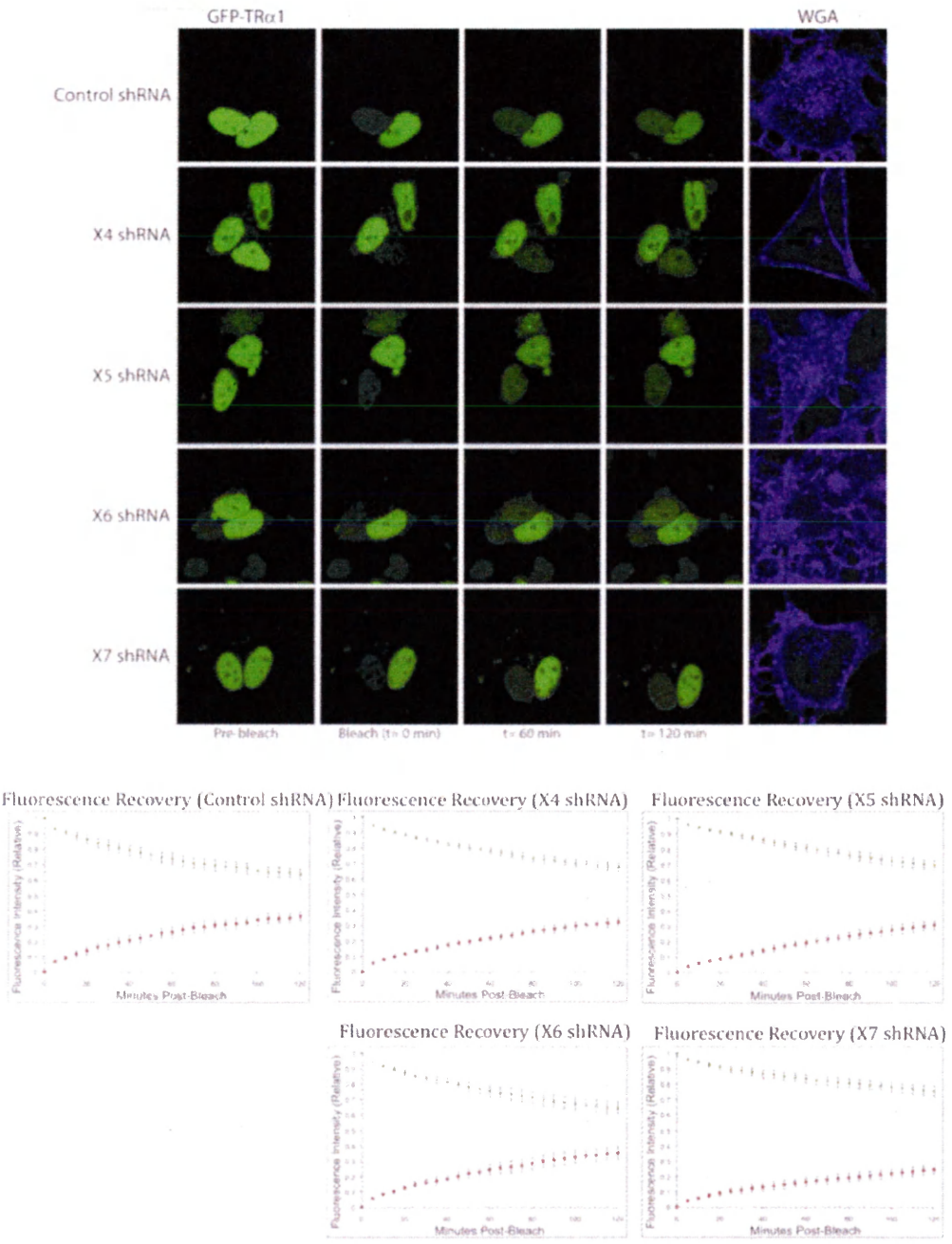


Figure 4. **Nuclear export and re-import of TRα1 is disrupted by knockdown of exportin 5 and exportin 7.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and shRNA expression plasmid targeting exportins 4, 5, 6, or 7 (X4, X5, X6, or X7 shRNA). A control shRNA expression plasmid (Control shRNA) was also used, where no protein knockdown occurs. Nucleocytoplasmic shuttling of TRα1 was monitored through FRAP (n=7, Control shRNA; n=7, X4 shRNA; n=9, X5 shRNA; n=7, X6 shRNA; n=9, X7 shRNA). The relative shuttling of GFP-TRα1 is indicated in the fluorescence recovery graphs where red squares are the intensity within bleached nuclei and green diamonds are the intensity within unbleached nuclei. Error bars indicate SEMs.

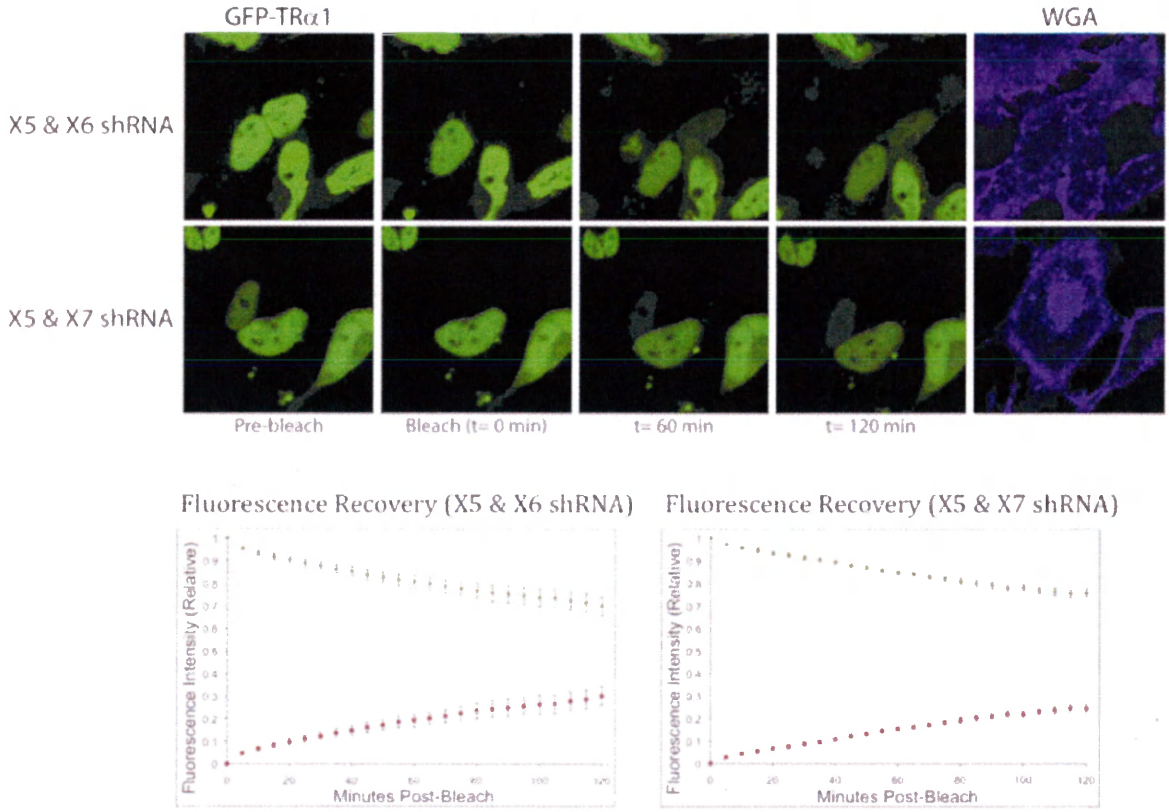


Figure 5. **Knockdown of both exportins 5 and 7 results in markedly slower shuttling kinetics of TRα1.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and shRNA expression plasmid targeting exportins 5 and 6 (X5 & X6 shRNA) or exportins 5 and 7 (X5 & X7 shRNA). FRAP experiments were performed to monitor the movement of GFP-TRα1 from the unbleached to the bleached nuclei (n=6, X5 & X6 shRNA; n=6, X5 & X7 shRNA). Fluorescence recovery graphs indicate the shuttling of GFP-TRα1 with red squares representing the relative intensity within bleached nuclei and green diamonds representing the relative intensity within unbleached nuclei. Error bars indicate SEMs.

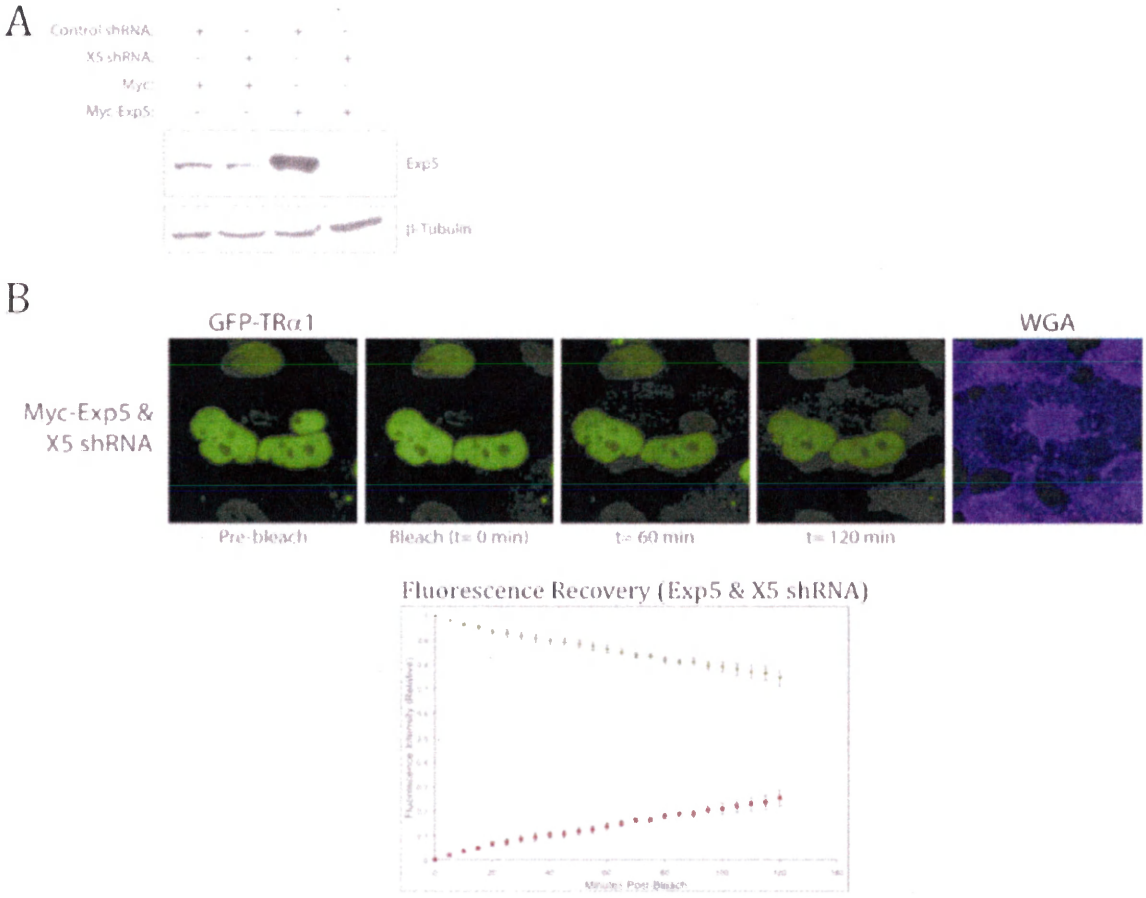


Figure 6. **Combined overexpression of exportin 5 and targeting exportin 5 with shRNA leads to highly efficient knockdown of exportin 5 and disrupts the shuttling of TRα1.** A. To determine knockdown of exportin 5 protein levels, HeLa cells were transiently transfected with GFP-TRα1 expression plasmid, Myc-tagged exportin 5 (Myc-Exp5) expression plasmid, Myc expression plasmid as a control, and shRNA expression plasmid targeting exportins 5 (X5 shRNA). Whole cell extracts were subject to Western blot analysis using anti-Exp5 antibodies and anti-β-tubulin antibodies, as a loading control. Bio-Rad pre-stained Kaleidoscope protein molecular mass standards were used to confirm protein identity (Exp5, 136 kDa; β-tubulin, 55 kDa). B. To determine how this highly efficient exportin 5 knockdown affected shuttling of TRα1, HeLa cells were transfected with GFP-TRα1 expression plasmid, Myc-Exp5 expression plasmid, and X5 shRNA expression plasmid. FRAP experiments were performed to monitor the nucleocytoplasmic shuttling of GFP-TRα1 (n=4). A fluorescence recovery graph indicating the shuttling of GFP-TRα1 was generated. Green diamonds represent relative fluorescence intensity within unbleached nuclei, while red squares represent intensity within bleached nuclei. Error bars indicate SEMs.

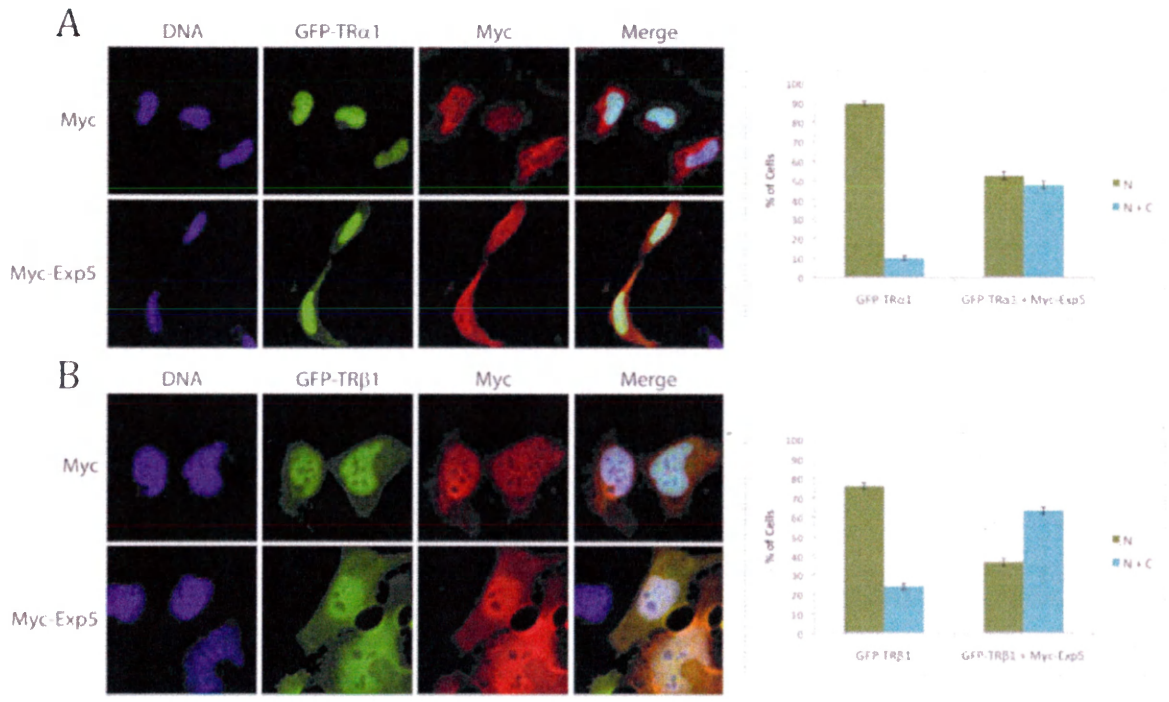


Figure 7. **Overexpression of exportin 5 promotes nuclear export of TR.** A. HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and Myc or Myc-Exp5 expression plasmid. After 17-19 hours, cells were fixed, immunostained with anti-Myc antibodies (red) and analyzed by fluorecence microscopy. Nuclei were stained for DNA with DAPI (blue). Bar graph summarizes the effect of overexpressing exportin 5 on TRα1 distribution in two categories; nuclear (N), nuclear and cytoplasmic (N + C). Error bars represent SEMs. Chi-square, $p<0.001$ (n=4 with 3 replicates each, at least 100 cells per replicate) B. Parallel experiments were performed with HeLa cells transfected with GFP-TRβ1 expression plasmid and Myc or Myc-Exp5 expression plasmid. Bar graph summarizes the effect of overexpressing exportin 5 on TRβ1 subcellular distribution. Error bars represent SEMs. Chi-square, $p<0.001$ (n=3, 3 replicates each, at least 100 cells per replicate)

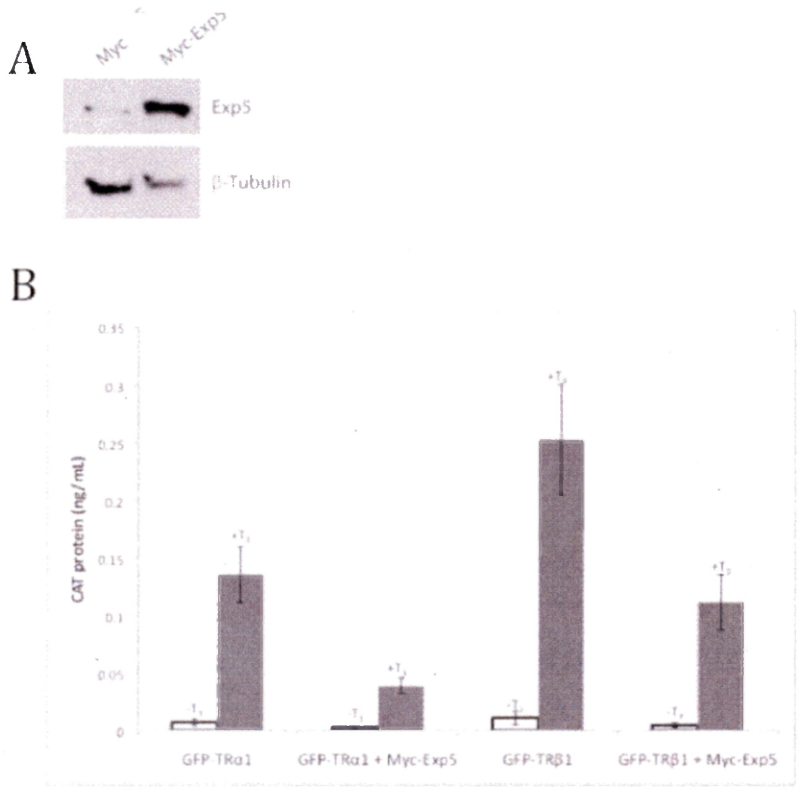


Figure 8. **Overexpression of exportin 5 decreases TR-mediated gene expression.** A. Exportin 5 was overexpressed in HeLa cells. Whole cell extracts of cells transfected with Myc or Myc-Exp5 expression plasmid were subject to Western blot analysis using anti-Exp5 and anti-β-tubulin antibodies. B. HeLa cells were transiently transfected with GFP-TR (α1 or β1) expression plasmids, Myc or Myc-Exp5 (exportin 5) expression plasmids, and tk-TREp-CAT reporter plasmids. Twelve hours after adding 100 nM T_3, CAT ELISAs were performed using whole cell extracts to determine the CAT protein levels. Reporter gene expression is measured in ng/mL and determined from CAT protein levels by ELISA (n=4). Basal levels of reporter gene expression in HeLa cells transiently transfected with GFP-TR (α1 or β1) expression plasmid and CAT-Basic Vector expression plasmid showed no CAT protein levels (data not shown). Error bars indicate SEMS.

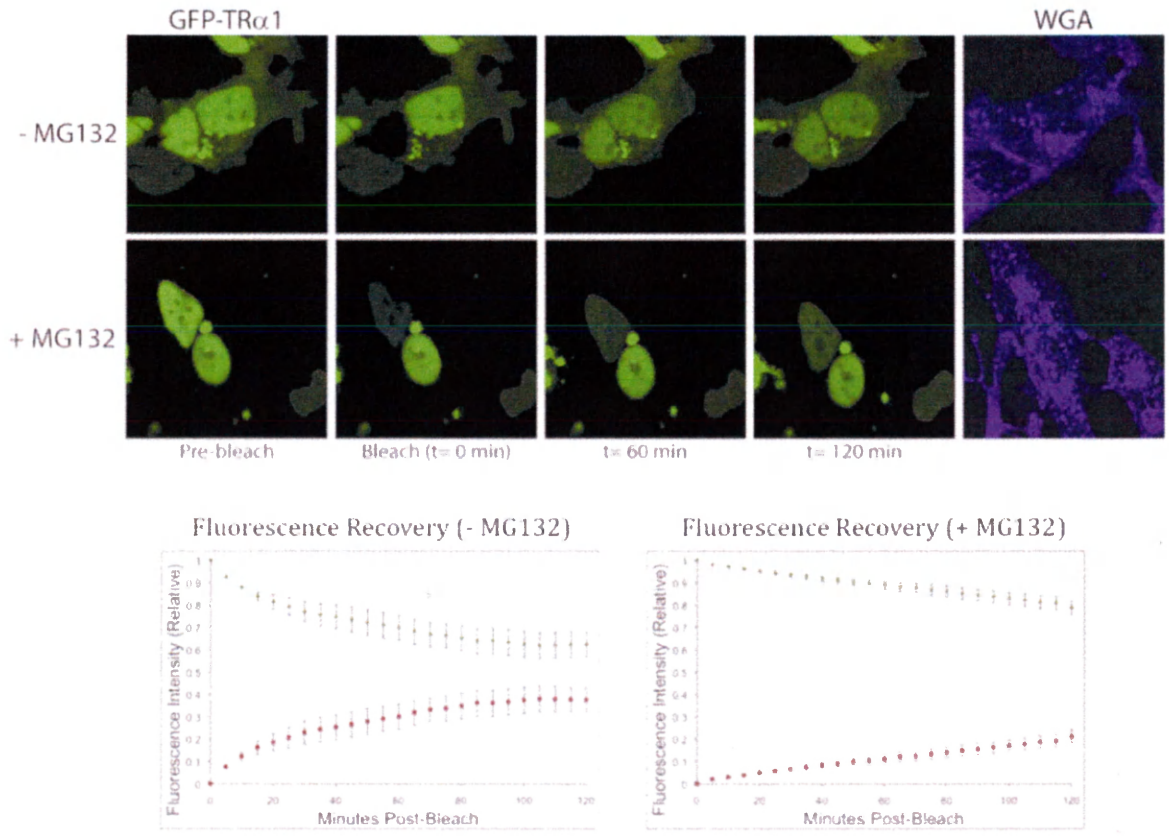


Figure 9. **Proteasome inhibition disrupts nucleocytoplasmic shuttling of TRα1.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmid. FRAP experiments were performed in multinucleated cells in the presence or absence of MG132 to monitor the nucleocytoplasmic shuttling of GFP-TRα1 (n=4, - MG132; n=4, + MG132). A fluorescence recovery graph indicating the shuttling of GFP-TRα1 was generated. Green diamonds represent relative fluorescence intensity within unbleached nuclei, while red squares represent intensity within bleached nuclei. Error bars indicate SEMs.

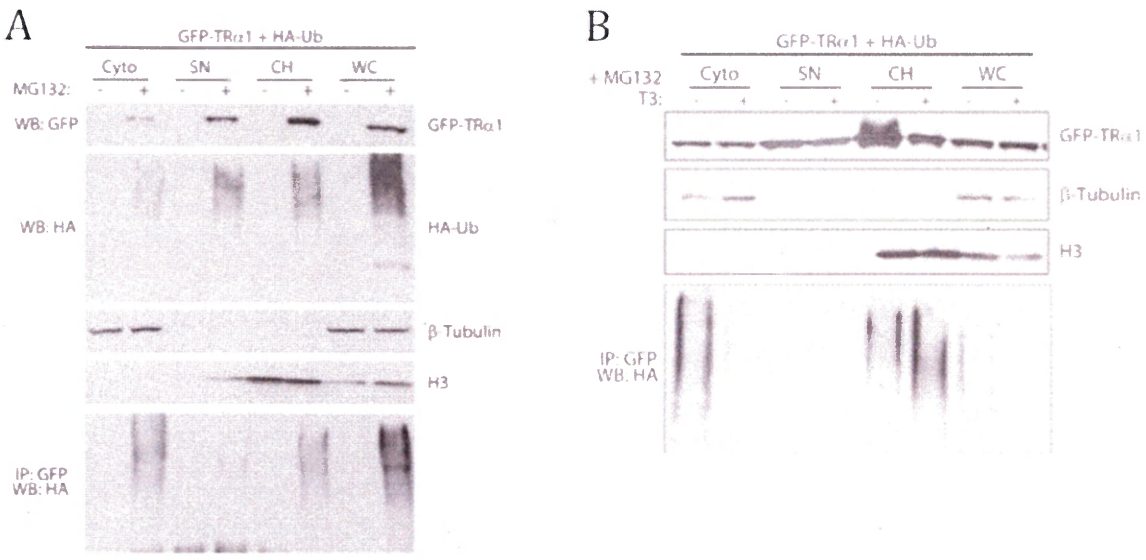
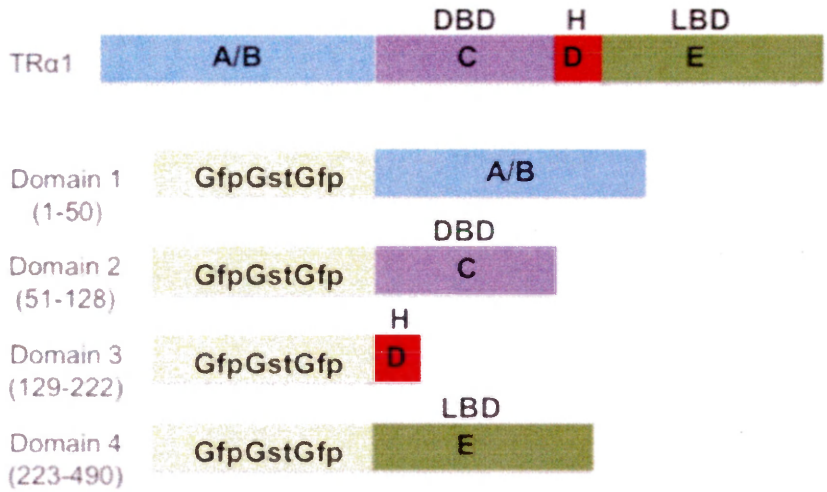


Figure 10. **Ubiquitinated TRα1 localizes to the cytoplasm and chromatin.** HeLa cells were transiently transfected with GFP-TRα1 and HA-Ub expression plasmids and incubated with MG132 (A) or with MG132 and T_3 (B) for 14-16 hours prior to fractionations. Whole cell extract and fractionations (Cyto: cytoplasmic proteins, SN: soluble nuclear proteins, CH: chromatin-bound proteins, WC: whole cell proteins) were subject to Western blot analysis. Cytoplasmic protein β-tubulin and nuclear protein (primarily chromatin-bound) Histone H3 served as controls to ensure there was no cross-compartment contamination in fractionations (β-tubulin, 55 kDa; H3, 17 kDa). TRα1 was immunoprecipated using anti-GFP antibody. The ubiquitinated status of TRα1 was then analyzed by western blot using anti-HA antibody. TRα1 was degraded through the ubiquitin-proteasome pathway. A. With MG132, ubiquitinated unliganded TRα1 was localized to the cytoplasm and chromatin. (n=3) B. With MG132 and T_3, ubiquitinated liganded TRα1 was localized to primarily the chromatin. Whole cell extract from the same experiment is also shown with a longer exposure time to show ubiquitinated TR. Whole cells extracts from cells transfected with GFP-TRα1 and Myc is used as a control. (n=2)

A



B

1 meqkpskvec gsdpeensar spdgkrkrkn gqcslktsms gyipsyldkd eqcvvcgdka
61 tgyhyrcitc egckgffrrt iqknlhptys ckydsccvid kitmqcqlc rfkkciavgm
121 amdlviddsk rvakrklieq nrerrrkeem irslqqrpep tpeewdlihi ateahrstna
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241 elpcedqiil lkgccmeims lraavrydpe sdtltlsgem avkreqlkng glgvvsdaif
301 elgksisafn lddtevallq avllmstdrs gllcvdkiek sqeayllafe hyvnhrkhni
361 phfwpkllmk erevqssily kgaaaegrpg gslgvhpegq qllgmhvvqg pqvrqleqql
421 geagslqgpv lqhqspkspq qrllellhrs gilharavcg eddsseadsp ssseeepevc
481 edlagnaasp

Figure 11. **A/B domain, Hinge domain, and LBD of TRα1 have potential sites for ubiquitination.** A. Schematic representation of individual domain constructs of TRα1 tagged with GFP-GST-GFP (made by Dr. Mavinakere). B. Domains 1 (A/B domain), 3 (Hinge domain), and 4 (LBD) have potential lysine residues that may be ubiquitinated according to UbPred, an online predictor of potential ubiquination sites.

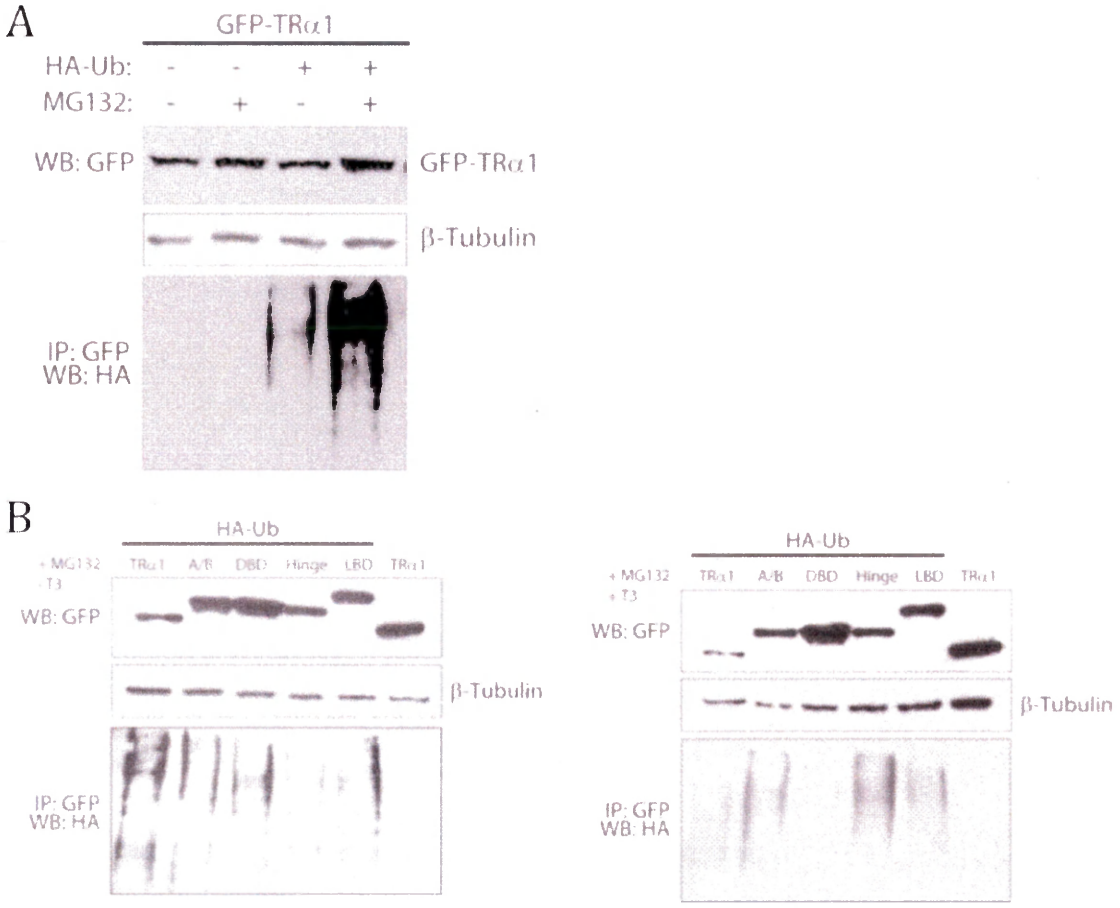

Figure 12. **Ligand-dependent and independent ubiquitination of TRα1 domains.** A. To show that TRα1 is directly ubiquitinated, HeLa cells were transfected with GFP-TRα1 expression plasmid and HA-Ub (ubiquitin) or Myc expression plasmid and inbucated with DMSO or MG132. Immunoprecipitation with anti-GFP antibody was performed with whole cell extracts that were lysed after 14-16 hours. Immunopreciptation samples and whole cell extracts were subject to Western blot analysis with anti-GFP, anti-HA, anti-β-tubulin antibodies, as an internal control. (n=3) B. To determine which TRα1 domains are ubiquitinated, HeLa cells were transiently transfected with GFP-GST-GFP-TRα1 domain expression plasmids (A/B, DBD, Hinge, LBD) and HA-Ub expression plasmid with and without MG132 and T_3 for 14-16 hours. Whole cell extracts were subject to Western blot analysis after immunoprecipitation with anti-GFP antibody. Whole cell extract from HeLa cells transfected with GFP-TRα1 expression plasmid and Myc was used as a control. (n=2, -T_3; n=2, +T_3)

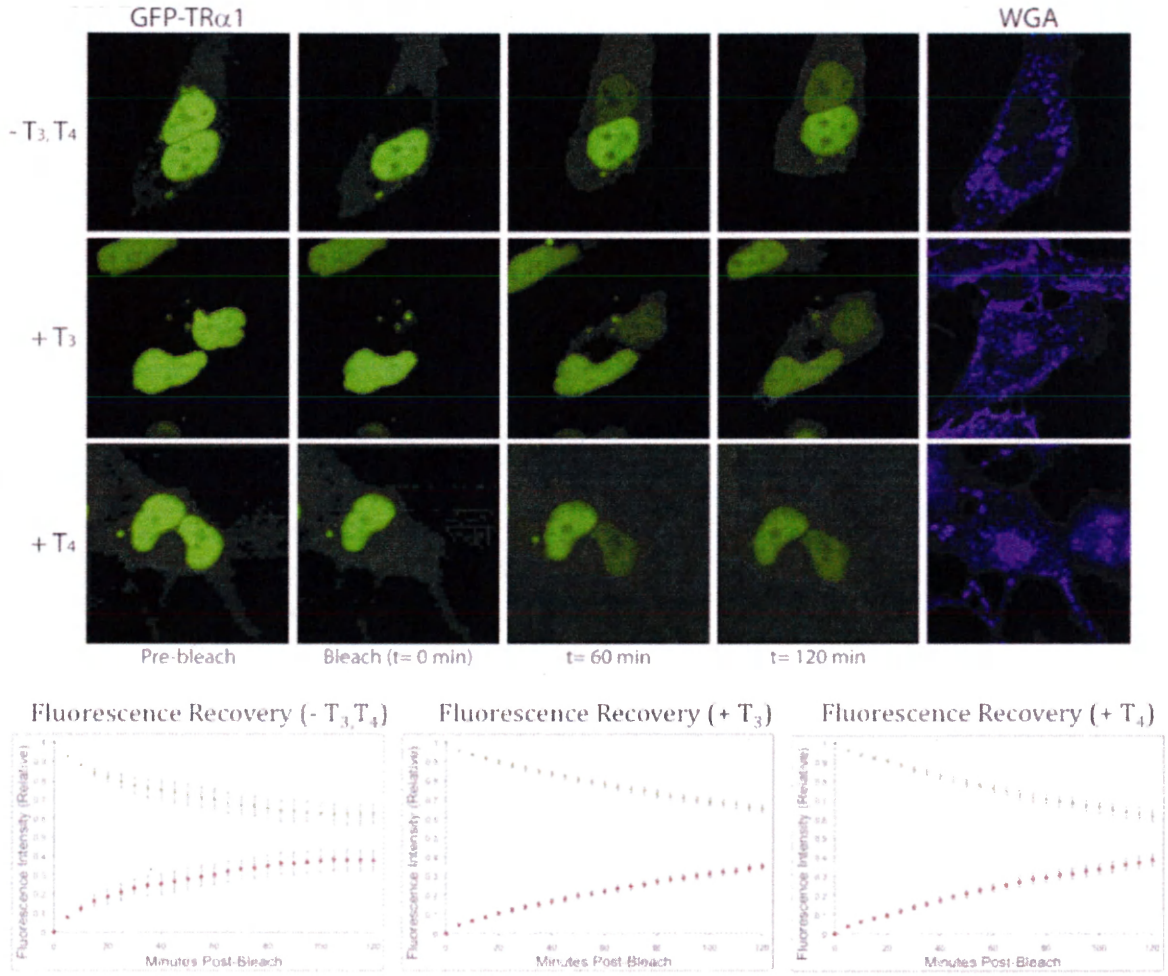

Figure 13. **Addition of T_4 promotes TRα1 shuttling.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmid. Cells were incubated with 100 nM T_3 or 100 nM T_4 prior to and during experiments. FRAP was performed to monitor the nucleocytoplasmic shuttling of GFP-TRα1 (n=8, T_3; n=6, T_4). A fluorescence recovery graph indicating the shuttling of GFP-TRα1 was generated. Green diamonds represent relative fluorescence intensity within unbleached nuclei, while red squares represent intensity within bleached nuclei. Error bars indicate SEMs.

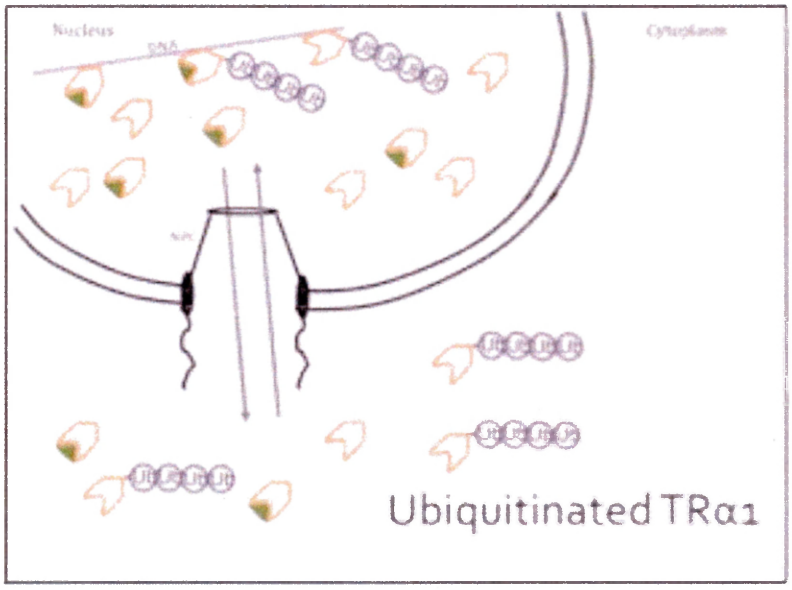

Figure 14. Model of ligand-independent and ligand-dependent TR ubiquitination. TRα1 rapidly shuttles in and out of the nucleus in the presence or absence of ligand. When TR is not bound to ligand, it can be ubiquitinated in the cytoplasm and while bound to chromatin. However, when ligand is present, liganded TR is ubiquitinated only while bound to chromatin.

CHAPTER 3: OTHER EXPERIMENTAL RESULTS

Since TRs can utilize multiple export factors in their nuclear export pathway, we sought to inhibit multiple exportins mediating TR shuttling. Parallel FRAP experiments utilizing RNAi were performed in the presence of LMB to block CRM1-mediated export. The data from these experiments give rise to more questions than answers; and therefore, continued research is needed in order to fully understand how inhibiting multiple export pathways impacts protein shuttling.

RESULTS

Blocking CRM1 and knocking down exportins do not fully disrupt nucleocytoplasmic shuttling of TRα1. TR has multiple NESs characterized in the ligand binding domain and can utilize multiple export pathways. To test if nuclear export can be completely blocked, we examined the kinetics of TR nuclear export when inhibiting both the CRM1-dependent and CRM1-independent pathways. Since we showed that exportin 5 mediates nuclear export of TRα1, we predicted that inhibiting CRM1 with LMB and knocking down exportin 5 would greatly disrupt the nucleocytoplasmic shuttling. HeLa cells co-transfected with GFP-TRα1 and shRNA expression plasmids (targeting exportin 5, 6, or exportin 5 and 6) were incubated with LMB prior to and during FRAP experiments.

CRM1 was shown to inhibit nuclear export and re-import of TRα1, consistent with previously reported observations by Grespin et al. (2008). Recovery of fluorescence to bleached nuclei within live cells transfected with a

control shRNA in the presence of LMB was 16% (±2%) at 60 min and 40% (±4%) at 120 min (Fig. 15). Surprisingly, when inhibiting CRM1 and knocking down exportin 5, the kinetics of TRα1 nuclear export were not as slow as when blocking CRM1 alone. In the presence of LMB and knockdown of exportin 5, recovery of fluorescence to bleached nuclei was measured at 33% (±2%) equilibration at 60 min and 53% (±2%) equilibration at 120 min (Fig. 15). Compared to knockdown of exportin 5 alone, disrupting both pathways caused a markedly slower nuclear export and re-import where fluorescence recovery was measured at 60 min and 120 min at 38% (±2%) equilibration and 61% (±3%) equilibration. This observation that nuclear export was not completely blocked was not expected; parallel experiments were performed to knockdown exportin 6, or exportin 5 and 6, as well as inhibiting CRM1. These experiments showed similar results with recovery of fluorescence in bleached nuclei equilibrated for 60 and 120 min at 64% (±4%) and 88% (±5%), respectively for exportin 6 and 30% (±3%) and 50% (±3%), respectively for exportin 5 and 6 knockdown (Fig. 15). However as expected, knocking down exportin 6 and inhibiting CRM1 did not show as great a disruption in nuclear export compared to the other conditions.

Since the kinetics of TRα1 nuclear export were not disrupted as greatly in comparison to inhibiting CRM1 alone, we examined how knocking down transportin 1, which was shown to not be involved in TR nuclear export, and inhibiting CRM1 with LMB would affect shuttling kinetics (Fig. 15). Nuclear export kinetics of TRα1 when inhibiting CRM1 and knocking down transportin 1 was similar to the kinetics when inhibiting CRM1 alone. Fluorescent recovery to

bleached nuclei equilibrations at 60 and 120 min were measured at 62% (±6%) and 76% (±3%) for knocking down transportin 1 (see Fig. 3) while in the presence of LMB fluorescence recovery was measured at 16% (±2%) and 40% (±4%) for 60 and 120 min , respectively (Fig. 16). When comparing these data with exportin 6 knockdown and CRM1 blocked, the kinetics were not the same and were markedly slower for transportin 1. This may be due to off-target effects of knocking down an exportin in comparison to knocking down a transportin.

Knocking down exportin gene expression causes no significant effect on T_3-mediated genes. Since we previously showed that overexpressing exportin 5 causes a decrease in T_3-mediated gene expression, we examined the transcriptional activity of GFP-TRα1 when knocking down exportin 5 using a CAT reporter under control of a positive TRE. We predicted that knocking down exportin 5 would cause an increase in T_3-mediated genes since nuclear export of TR would be partially inhibited. HeLa cells were transiently transfected with GFP-tagged TRα1 expression plasmids, shRNA control or shRNA expression plasmid targeting exportin 5, and tk-TREp-CAT reporter plasmids. Twelve hours after adding 100 nM T_3, CAT ELISAs were performed using whole cell extracts to determine CAT protein levels. The levels of CAT protein produced were not consistent for each experiment for exportin 5 knockdown. Exportin 5 knockdown at the protein level was previously shown to not be as efficient (Fig. 17); this may also explain why only a slight increase in CAT protein production was seen. These data may also provide evidence that the slight increase in T_3-responsive

gene expression occurs due to another level of transcriptional control not accounted for, other than nuclear export.

DISCUSSION

Previously, we showed that TRs have multiple shuttling pathways, CRM1-dependent and CRM1-independent (Grespin et al., 2008; Mavinakere et al., submitted). *In vivo* approaches of live cell imaging and FRAP were used to inhibit multiple nuclear export pathways at one time to determine how TR shuttling was impacted. The CRM1-mediated nuclear export pathway was inhibited with LMB while various exportins were knocked down. From our data, we saw that TRα1 shuttling kinetics were not fully disrupted when multiple pathways were inhibited. During the first hour of the experiment, nucleocytoplasmic shuttling of TRα1 was disrupted similar to the control where only CRM1 was inhibited. This observation may be due to off-target effects of knocking down exportins since these proteins are essential to cell survival. As another control, TR shuttling when transportin 1 was knocked down and CRM1 inhibited was also examined. The discovery that transportin 1 knockdown and CRM1 inhibition caused a similar disruption to CRM1 inhibition alone supports that exportin knockdown causes other off-target effects. These data may also provide evidence that TRs can be flexible and may utilize other exportins available when multiple exportins are not present for nuclear export. TRs have multiple NESs, which may aid in ensuring that the protein may still be exported

from the nucleus. Continued research examining potential off-target effects of exportin knockdown and CRM1 inhibition is needed.

We also showed that knocking down exportin 5 does not cause a large increase in T_3-mediated gene expression. In contrast, we saw that overexpressing exportin 5 decreases T_3-mediated gene expression. Knocking down exportin 5 may not statistically increase T_3-mediated gene expression due to various reasons. Exportins are important for many macromolecules to be translocated out of the nucleus; thus, knockdown of these important proteins may cause other downstream effects. Another possiblity is that even though nuclear export is reduced, other transcriptional controls are still present within the cell that are not accounted for. Another important note to make is that more efficient knockdown of exportin 5 is seen when the same protein is overexpressed. Continued experiments examining how this more potent knockdown of exportin 5 affects gene transcription will be critical for further understanding.

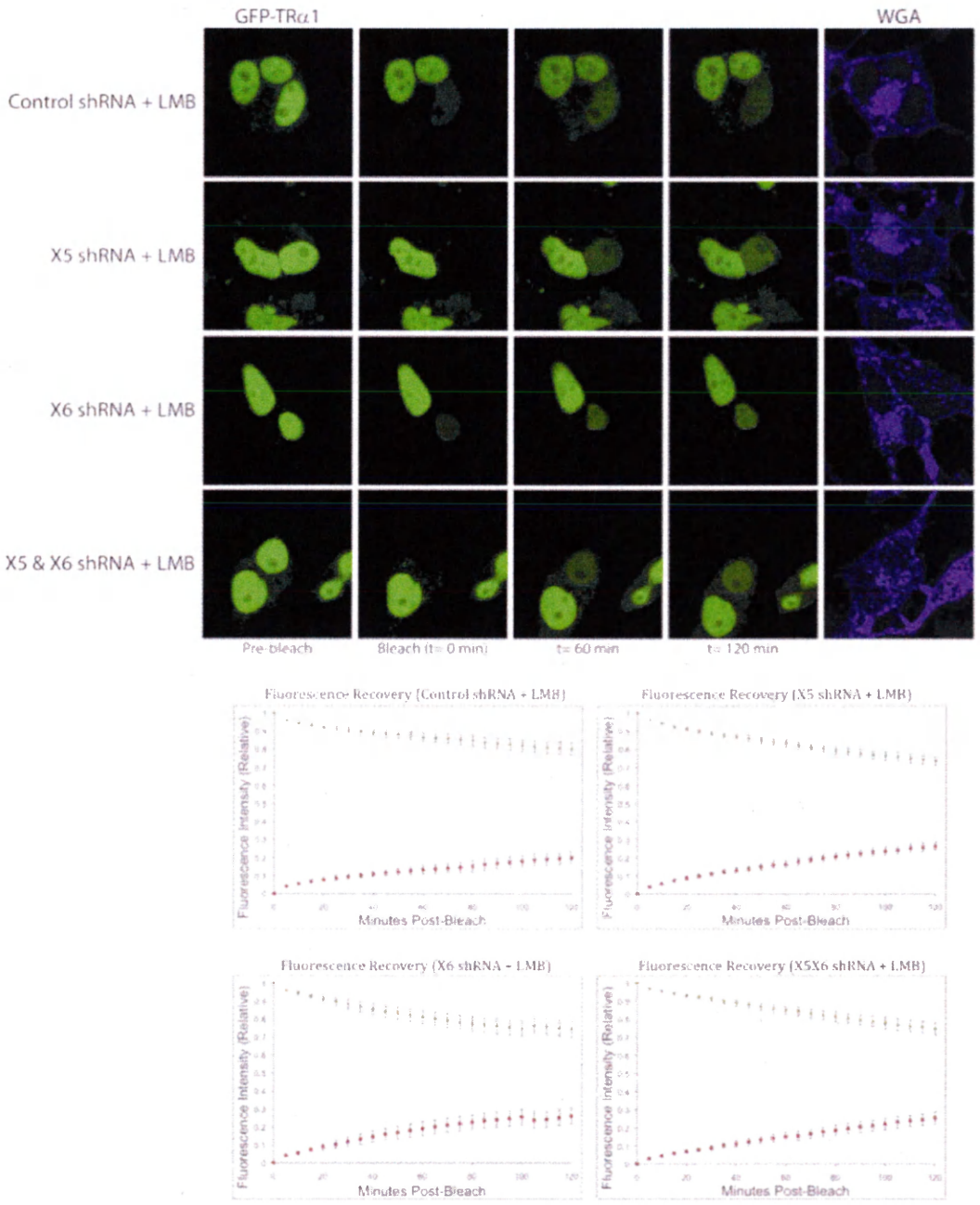

Figure 15. **Addition of LMB and knocking down exportins does not completely block shuttling of TRα1**. HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and shRNA expression plasmid targeting exportin 5, exportin 6, or both exportin 5 and 6 (X5, X6, or X5 & X6). A control shRNA expression plasmid was also used for a control where no gene expression knockdown occured. Prior to and during FRAP experiments, cells were incubated with LMB. FRAP experiments were performed in multinucleated live cells incubated to monitor the movement of GFP-TRα1 from the unbleached to the bleached nuclei (n=6, Control shRNA + LMB; n=5, X5 shRNA + LMB; n=8, X6 + LMB; n=6, X5 & X6 shRNA + LMB). Fluorescence recovery graphs showing the movement of GFP-TR in and out of the nucleus were generated with red squares representing the relative intensity within bleached nuclei and green diamonds representing the relative intensity within unbleached nuclei. Error bars indicate SEMs.

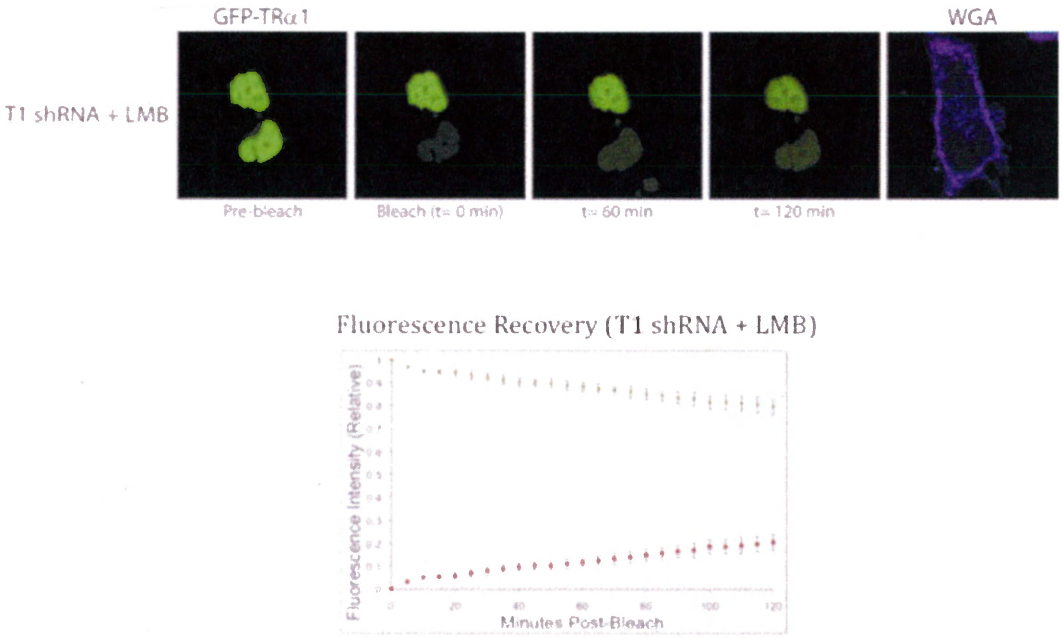

Figure 2. **Addition of LMB when knocking down transportin 1 affects shuttling of TRα1 similar to experiments with the addition of LMB when no gene expression knockdown occurs**. HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and shRNA targeting transportin 1 knockdown (T1). FRAP experiments were performed to monitor the nucleoplasmic shuttling of TRα1 (n=5). Fluorescence recovery graph was generated with red squares indicating the relative intensity within bleached nuclei and green diamonds indicating the relative intensity within unbleached nuclei. Error bars indicate SEMs.

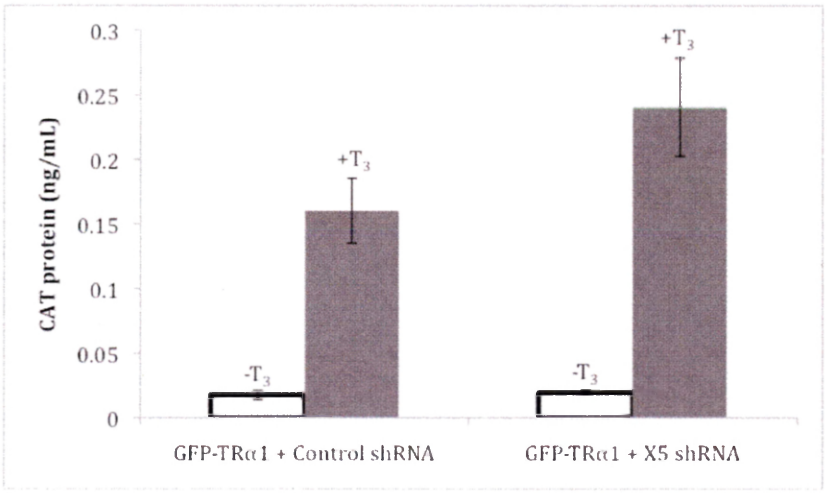

Figure 3. **Exportin 5 knockdown slightly increases TR-mediated gene expression.** HeLa cells were transiently transfected with GFP-TRα1 expression plasmids, shRNA expression plasmid (control or targeting exportin 5), and tk-TREp-CAT reporter plasmids. Twelve hours after adding 100 nM T_3, CAT ELISAs were performed using the whole cell extracts to determine the CAT protein levels. Reporter gene expression is measured in ng/mL and determined from CAT protein levels by ELISA. Basal levels of reporter gene expression in HeLa cells transiently transfected with GFP-TRα1 expression plasmid and CAT-Basic Vector expression plasmid showed no CAT protein levels (data not shown). Error bars indicate SEMS.

CHAPTER 4: GENERAL DISCUSSION

Research reported in this thesis provides strong evidence that exportins 5 and 7 play a key role in mediating the CRM1-independent nuclear export of TRα1. While previous research shows that proteasome inhibition blocks TR degradation through the ubiquitin proteasome-mediated pathway; we also show that proteaseome inhibition blocks TR mobility. These data show a direct interaction of ubiquitin with multiple domains of TR. TRα1 can be ubiquitinated in the A/B domain and LBD in the presence or absence of T_3; however, the DBD and Hinge domain can also be ubiquitinated depending on the presence of ligand. While T_3 promotes more rapid TR degradation, it does not promote more rapid nuclear export. In contrast, T_4 promotes more rapid nuclear export and re-import of TR. Interestingly, T_3 does impact the localization of ubiquitinated TR. In the presence of the proteasome inhibitor MG132, ubiquitinated TR accumulates in the cytoplasm and chromatin; conversely, with MG132 and T_3, ubiquitinated TR is primarily chromatin-bound and does not accumulate in the cytoplasm. As a whole, this research provides insight into the mechanism and physiological significance of TR nuclear export.

FUTURE DIRECTIONS

All FRAP and RNAi coupled experiments were completed in the absence of ligand. Even though nuclear export did not change drastically in the presence of 100 nM T_3, nuclear export increased when 100 nM T_4 was present. T_3 has been shown to cause a conformational change in TR, primarily in helix 12

of the ligand binding domain, which also has a NES present. Here, we have assumed that the presence of T_3 is not changing the NES that may be utilized for nuclear export and that any NES within TR is affected with exportin knockdown. RNAi and FRAP experiments in the presence of ligand would be an interesting approach to answer the question of whether there is a ligand-dependent NES changing the exportin utilized in the nuclear export mechanism. Ligand-dependent NESs have been seen in other NRs such as GR and VDR (Prufer and Barsony, 2002), which would support the potential that TR may behave similarly.

Continued research will also be needed to address protein:protein interactions of these exportins with TR in order to identify exportins that directly bind this receptor. An *in vivo* approach using HeLa cells and co-immunoprecipitations can be utilized to investigate protein:protein interactions. Since protein transport is a transient event, it may be difficult to co-immunoprecipiate TR with an export factor; however, use of a Ran-GTP mutant unable to hydrolyze to Ran-GDP could promote TR nuclear export and binding to export factors. Non-hydrolyzable Ran-GTP inhibits cargo disassembly since Ran-GTP promotes export and would enhance the binding of exportins to their cargo (Kazgan et al., 2010; Mingot et al., 2004). The benefit of this experiment would be that it is performed *in vivo* and could be continued with future experiments examining the conditions needed to promote export complex formation. Alternatively, an *in vitro* approach utilizing pull-down assays can also be used to address protein:protein interactions of these exportins with TR.

This experimental approach examining protein:protein interactions can also test what exportins bind particular NESs of TRs. Previously in the lab, two CRM1-independent NESs have been characterized in helix 6 and 12 of the TR ligand binding domain (Mavinakere et al., submitted). The same approach performing co-immunoprecipitations with HeLa cells transiently transfected with the mutant RanQ69L, a nonhydrolyzable Ran, and TRα1 NES mutants (already made by Dr. Mavinakere) can be taken. Although it is already known that CRM1 can bind to a leucine rich NES, it is not known what NES other export factors bind. Determining the NES sequences that exportins bind will aid in understanding the mechanism by which NR, and other proteins exit the nucleus. Also comparing nuclear export kinetics of NES mutant constructs with exportin 5 and 7 knockdown would be another approach to reveal whether a NES may potentially utilize one exportin over another.

Since overexpression of exportin 5 was utilized in this study, it can be predicted that overexpressing exportin 7 would also impact TR localization. Thus, overexpressing exportin 7 to examine TR subcellular distribution, and also overexpressing multiple exportins would be interesting to examine to determine if TR localization could be completely changed by the presence of multiple exportins mediating nuclear export. Investigating whether overexpressing both exportin 5 and 7 would cause complete or nearly complete cytoplasmic TR localization may be another future direction of this research.

Another focus of this thesis was examining the physiological significance of TR nuclear export. Previously, TR ubiquitination and how it impacts nuclear

export had not been examined extensively; however, researchers reported variable results on the impact of ubiquitination on NR nuclear export. As it was shown that multiple domains of TRα1 have ubiquitination sites, mutagenesis analysis combined with immunoprecipitations would be needed in order to determine the exact lysines in TR that are ubiquitinated. These experiments also need to be performed in the full length protein since mutliple domains may work together to direct ubiquitination of TR. Constructs with multiple TR domains may also be used in immunoprecipitations since Dace et al. (2000) showed that the LBD is the most important domain needed for TR degradation. These immunoprecipitation assays could examine if degradation correlates with ubiquitination, since this research shows that any domain may be ubiquitinated. Since the understanding of how ubiquitination affects transcription has only recently emerged, how ubiquitination impacts TR transcriptional control would also be an area of interest for future work. These experiments could utilize a CAT ELISA with TR mutants unable to be ubiquitinated and in the presence of the proteasome inhibitor MG132 at varying time points. FRAP experiments were performed with cells incubated in MG132 for up to 6 hours; in contrast, cells for ubiquitination experiments were incubated in MG132 for over 14 hours. FRAP experiments with cells incubated in MG132 for this time span may be of importance in understanding how MG132 affects TR and ubiquitinated TR mobility.

CONCLUSION

With the discovery that TRs shuttle between the nucleus and cytoplasm, a novel mechanism for T_3-mediated gene regulation emerged. The observation that T_3-mediated gene expression is altered when normal TR shuttling is disrupted directly supports the importance of shuttling. In addition, when these receptors are mutated, they can disrupt homeostasis and promote tumor growth and proliferation. While the exact mechanisms of shuttling for NRs in the superfamily remain to be elucidated, TRs were previously shown to follow a CRM1/calreticulin pathway and other uncharacterized CRM1-independent pathways (Grespin et al., 2009; Mavinakere et al., submitted). Other NRs have also been shown to utilize CRM1 and exportin 5, which is a novel export pathway for these proteins. Here, we showed that TRs can follow novel export pathways mediated by exportin 5 or exportin 7. Further analysis of nuclear export pathways and the NES sequences mediating this export will continue to give rise to the mechanism involved in T_3-mediated gene regulation.

Although this thesis research has shed some light on the significance of TR nuclear export, the physiological significance of export is not fully understood. Some research has shown that TRs are modified by signals from the cytoplasm, which may account for this export (Guigon and Cheng, 2009). For many proteins, post-translational modifications have also been shown to affect protein localization (Ikeda and Kerppola, 2008; Shank et al., 2008). TRs utilize the ubiquitin-proteasome mediated degradation pathway similar to all NRs. Current research lacks in the impact of ubiquitination on nuclear export; thus, we focused

on elucidating where ubiquitinated TR localizes and how it impacts TR export. Ubiquitinated TR was found to change subcellular localization when ligand was present. Similar to estrogen receptors (Tateishi et al., 2004), this finding supports that TRs may be ubiquitinated in two separate locations depending on presence of ligand. T_3 only slightly slowed nuclear export of TR. Thus, our data point to the conclusion that ubiquitination of TR only plays a small role in nuclear export.

In conclusion, this thesis research has implications for T_3-mediated gene regulation at both the level of TR nuclear export and post-translational modification. While protein mislocalization is seen in various cancers (Bonamy et al., 2005; Bunn et al., 2001), our findings that exportin availability impacts TR localization, indicates that additional research is needed in exportin involvement in cancer and endocrine disease. Currently, research lacks in exportin-related diseases, as they may present themselves as diseases related to their cargo mislocalization. Exportin 5 was suggested to be a therapeutic target for shRNA delivery (Yi et al, 2005); our research supports that exportin 5 overexpression may also create off-target effects where cargo is mislocalized and may cause cancer or endocrine disease due to misregulation of T_3-mediated genes, or other NR-mediated genes. Our research has novel implications for other NR nuclear export pathways where exportins 5 and 7 also may be utilized in their as yet uncharacterized CRM1-independent nuclear export pathway. Finally, the research presented in this thesis has shed light on the NR export field and has

implications for how misregulated NR-mediated gene expression may promote cancer and other endocrine diseases.

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