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Characterization of Thyroid Hormone Receptor Export Pathways

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CHARACTERIZATION OF THYROID HORMONE RECEPTOR EXPORT PATHWAYS

By: Hallie Nicole Nelson

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ABSTRACT

Thyroid hormone receptor (TR) is critical in many aspects of metabolic control and development. TR acts as a transcription factor when bound to thyroid hormone (T_3 and T_4) to change gene expression of thyroid hormone response elements (TREs). There are two main isoforms of TR: TR α and TR β . TR can shuttle into and out of the nucleus via an importin or exportin-mediated pathway through the nuclear pore complex. The importin or exportin binds to TR at a nuclear localization sequence (NLS) or a nuclear export sequence (NES) to transport TR into or out of the nucleus, respectively. A well characterized nuclear export pathway is the cooperative CRM1/calreticulin-mediated export pathway. However, TR α and TR β also can follow a CRM1-independent pathway for nuclear export. The main objective of this research was to determine if exportin 5 (XPO5) or RanBP17 mediate the export of TR via a CRM1-independent nuclear export pathway. XPO5 has been characterized as an export factor for microRNAs, as well as for the androgen receptor. RanBP17 has only been characterized as a potential exportin based on CRM1 homology. To determine if XPO5 mediates TR's export by the latter pathway, XPO5 was over-expressed in HeLa cells. The distribution and transcriptional activity of GFP-tagged TR α or TR β was analyzed by fluorescence microscopy and CAT ELISA, respectively. TR is primarily nuclear at steady-state, but TR had a more cytoplasmic distribution when XPO5 was overexpressed. In addition, CAT reporter gene expression under control of a thyroid hormone response element was markedly decreased when XPO5 was over-expressed, indicating less TR was present in the nucleus. TR has multiple nuclear export sequences (NESs) in the ligand-binding domain. We tested the helix 12 NES (NES-H12) and the NES located in the 3rd and 6th helices (NES-H3/H6) on their interactions with XPO5. The NES-H12 construct had a more cytoplasmic distribution when XPO5 was over-expressed, suggesting that XPO5 utilizes NES-H12 during export. We also over-expressed RanBP17 in HeLa cells and analyzed any changes in distribution of GFP-TR α or TR β , when analyzed by fluorescence microscopy and CAT ELISA, respectively. Our microscopy results suggest that RanBP17 facilitates nuclear export of TR β but not TR α , while the CAT ELISA results were inconclusive. In conclusion, our results suggest that XPO5 can mediate TR nuclear export via the NES located in helix 12 of the ligand binding domain. Further, RanBP17 may play a role in nuclear export of TR β ; however, replicate experiments are required to confirm this finding. Investigation of the role of other exportins, such as RanBP16/XPO7, is needed to fully characterize the multiple export pathways followed by TR. Further insight gained from the regulation of nuclear export of TR will ultimately enhance understanding of regulation of thyroid hormone-responsive gene expression and the role of TR in growth and development.

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INTRODUCTION

OVERVIEW

Thyroid hormone receptors (TR) are proteins that are members of the nuclear receptor (NR) superfamily. There are two main isoforms of TR: thyroid hormone receptor alpha (TR α) and thyroid hormone receptor beta (TR β). These transcription factors regulate gene expression by binding to their respective ligands and to target genes in the nucleus. Nuclear receptors move from the cell cytoplasm into the nucleus through the nuclear pore complexes (NPC). This process of moving in and out of the nucleus is called nucleocytoplasmic shuttling. There are two main signals for proteins to be imported or exported from the nucleus: nuclear localization sequences (NLS) and nuclear export sequences (NES). These signals act like a zip code for sending a protein to a specific location inside the cell (Weis, 1998). Highly conserved NES have been characterized for TR α . These NES are located at the 12th helix (NES-H12) and between the 3rd and 6th helices (NES-H3-H6) (Mavinakere et al., 2012). Protein movement into and out of the nucleus through the NPC is directed by other proteins called importins and exportins, respectively. One export pathway that is well characterized and used by many NRs is the CRM1-dependent pathway. CRM1 is characterized as an exportin that binds to a leucine-rich NES on the NR (Grespin et al., 2008). TR has also been described as using a CRM1-independent export pathway. The specifics of which exportins are used by TR remain ill-defined. TR is known to be very important in many biological processes, such as metabolic control, heart rate control, and development. Disregulation of TR can also cause cancer and disease. By understanding how TR nuclear export is regulated, insight can be gained on how to treat TR-associated diseases.

NUCLEAR RECEPTORS

Nuclear receptors (NR) are proteins that act as transcription factors. There are at least 50 NRs already identified, with over 100 known to exist (McKenna and O'Malley, 2002; McKenna

et al., 1999). The NR superfamily is the largest known family of metazoan transcription factors (McKenna et al., 1999). To be a member of the NR superfamily, each NR must have the following domains: an amino-terminal activation domain, DNA-binding domain, hinge region, and a carboxyl-terminal domain containing a ligand-binding region. Each NR also has two activation functions, AF-1 in the amino-terminal domain and AF-2 in the carboxyl-terminal domain (McKenna and O'Malley, 2002). Within the NR superfamily, there are three types. Type I NRs are the classical steroid receptors, Type II include the thyroid hormone receptor (TR), and Type III include recently characterized receptors, also called orphan receptors. Unlike Type I NRs, Type II NRs can bind to DNA in the absence of their ligand and act as repressors that silence their target gene (McKenna et al., 1999). When unbound to their ligand, Type I NRs are sequestered by heat shock proteins (hsp70, hsp90) and immunophilins to help control the steroid signal (Kumar et al., 2006).

Depending on whether an NR is bound to its ligand or not, the NR can have a different subcellular localization. When ligand-bound, all NRs are localized in the nucleus to act as transcription factors. Within the nucleus, the active NRs congregate in 'nuclear foci' (Kumar et al., 2006). However, when the ligand is not present, intracellular NRs can be localized to the nucleus, the cytoplasm, or across the whole cell uniformly. For example, when glucocorticoid receptor (GR) and androgen receptor (AR) are not bound to their respective ligands, they have a primarily cytoplasmic distribution, while the mineralocorticoid receptor (MR) is located throughout the cell. In contrast, the progesterone receptor (PR) and estrogen receptor (ER) remain in the nucleus, even when their ligands are absent. Other types of NRs have recently been identified as localizing to the plasma membrane and mitochondria (Kumar et al., 2006). Within the superfamily, all of the NRs are specific to certain pathways and tissue types.

Therefore, even though there are similarities in homology across the superfamily, each NR needs to be studied individually to fully understand the signaling process.

THYROID HORMONE

The thyroid hormone (TH) is a key player in metabolism and growth in many different cell types. The process of producing thyroid hormone occurs along the hypothalamus-pituitary-thyroid axis and is regulated by negative feedback loops (Yen, 2001). The hypothalamus releases thyrotropin-releasing hormone (TRH), which goes to the anterior pituitary gland causing the release of thyroid-stimulating hormone (TSH). TSH travels to the follicular cells of the thyroid gland, where thyroid hormone is released at large across the body (Waung et al., 2012). There are two types of TH, triiodothyronine (T3) and thyroxine (T4), which are structurally homologous except for a fourth iodine on T4. T3 is the active form of the hormone, and binds with a ten-fold greater efficiency to TRs than T4 (Oetting and Yen, 2007). However, T4 is the pro-hormone that is predominantly circulating within an organism (Waung et al., 2012). T3 is created from the pro-hormone T4 through deiodinating enzymes D1 and D2 (Visser et al., 2007).

Originally, it was thought that TH moves into the target cell through diffusion, like transport of steroids, but recent research indicates otherwise. The majority of TH is transported across the plasma membrane by specific membrane transport proteins and non-amino acid specific transporters (Waung et al., 2012). Some of the specific membrane transport proteins previously characterized are OATP1C1, MCT8, and MCT10. OATP1C1 is localized in the brain, while MCT8 is seen across tissue types, most prominently in the liver and heart, and MCT10 is also seen across tissues, concentrated mostly in the intestine, kidney, liver, and placenta (Visser et al., 2007).

Once in the nucleus of the cell, TH can bind to the thyroid hormone receptor (TR) and change transcription patterns of specific genes. When TH binds to TR, TH causes a conformational change in helix 12 of the ligand-binding domain of TR, which changes how TR can interact with thyroid hormone response elements (TREs) in the DNA (Oetting and Yen, 2007). Usually if TR is unbound to TH, TR acts as a corepressor to transcription. In contrast, when TH is bound, TR in most cases enhances transcription. Other factors, including corepressors (N-CoR and SMRT) and the extent of acetylation of histones contribute to gene expression or repression as well (Lonard and O'Malley, 2007).

While TH can affect genomic transcription through the classical mechanism by binding to TR, TH is also involved in other signaling cascades. TH plays a role in lipid metabolism and homeostasis by activating rate limiting enzymes involved in fatty acid hepatic synthesis and triglyceride esterification (Cordeiro et al., 2013). While the classical mechanism is the focus of this thesis, it is important to remember the TH has far reaching effects within an organism, and therefore must be regulated closely in multiple cell types.

THYROID HORMONE RECEPTOR

The thyroid hormone receptors (TR) are proteins that have two main isoforms TR α and TR β . These two TRs are encoded by the genes THRA and THRB on human chromosomes 17q11 and 3p24, respectively (Jazdzewski, 2011). Through alternative splicing, six specific isoforms are made: TR α 1, TR α 2, TR α 3, TR β 1, TR β 2, and TR β 3. TRs are activated when they are bound to both T3 and specific TREs in the promoter region of a gene. However, TR α 2 and TR α 3 are unable to bind to T3 (Jazdzewski, 2011). TR α 2 is also called *c-erbAa-2* and may act antagonistically to inhibit TH action (Yen, 2001). TRs are tissue specific in their expression. For example, TR α 1 is most predominantly expressed in skeletal muscle and brown fat, while

TR β 1 is seen most commonly in the brain, kidney, and liver (Yen, 2001). TR expression is widespread throughout the body, but certain isoforms have highly specialized tissue types. For instance, TR β 2 is only seen in the anterior pituitary gland and parts of the hypothalamus (Yen, 2001).

DOMAINS

TR has multiple domains that include the N-terminal domain, the DNA-binding domain (DBD), the hinge domain, and the carboxyl-terminal ligand-binding domain (LBD). While the N-terminal transactivation domain varies between isoforms, the DBD is highly conserved (Cheng et al., 2010). Each domain has a specialized function. The N-terminal domain harbors the amino-terminal activation function (AF-1), which aids in transcription activation. The DBD contains two zinc finger motifs, which bind TREs and are conserved across NRs (Cheng et al., 2010).

The hinge region originally was thought to function solely as a domain to separate the DBD and LBD. However, previous research has shown that TR has a nuclear localization signal (NLS) in the hinge region, along with another NLS only present in the N-terminal domain of TR α 1 (Mavinakere et al., 2012). A NLS is a sequence that tags a particular protein to get transported into the nucleus. Likewise a nuclear export sequence (NES) tags a protein for export from the nucleus. While many NRs, including the glucocorticoid receptor and androgen receptor, have their NLS in the DBD or LBD domain, surprisingly, TR has multiple NESs located in the LBD region, one at helix 12 and two more NES located between helix 3 and helix 6 (Mavinakere et al., 2012).

The LBD and DBD also have regions of dimerization. TR forms either a homodimer, or a heterodimer with the retinoid X receptor (RXR) (Oetting and Yen, 2007). Many members of

the NR superfamily can heterodimerize with RXR. When RXR is present, it allows for more nuanced transcriptional activity (Yen et al., 2006), through the ligand-sensitivity of RXR (Zhang et al., 1992). The LBD is also bound to heat shock proteins and other regulatory proteins when not bound to T3 (McKenna et al., 1999).

Each of the isoforms of TR has a slightly different amino acid sequence, and therefore, a slightly different structure. The THRA gene creates two different mRNAs and proteins through alternative splicing. TR α 1 and TR α 2 have the same 1-370 amino acid residues but differ in their LBDs after amino acid 370 (Oetting and Yen, 2007). Alternatively, the THRB gene has alternative promoter regions, which create two separate proteins, TR β 1 and TR β 2. TR β 1 and TR β 2 are structurally identical except for their N-terminal domains (Oetting and Yen, 2007). Since all the isoforms are slightly different structurally, and each domain has multiple functions, it is imperative to characterize each TR isoform individually, while looking at similarities across the group.

LOCALIZATION AND SHUTTLING

Under normal physiological conditions, the majority of TR is localized to the nucleus of the cell. TR α has an approximately 90% nuclear localization, while TR β has a slightly more cytosolic distribution, with approximately 85% nuclear localization. This partially cytosolic distribution of TR lead researchers to investigate a seemingly constant flux of TR into and out of the nucleus, also called shuttling (Baumann et al., 2000; Bunn et al., 2001). This equilibrium is a balancing act between nuclear import, nuclear retention, and nuclear export.

Transport of proteins into and out of the nucleus through the nuclear pore complexes (NPC) is mediated by transport receptors (Gwizdek et al., 2004). The NPC is made up of proteins called nucleoporins, which span the nuclear envelope to create an aqueous channel

between the nucleus and cytoplasm (Brownawell and Macara, 2002). Small molecules (<60 daltons) can diffuse through the NPC, but large molecules move through facilitated by transport receptors that are members of the karyopherin β family (Gwizdek et al., 2004). The transport receptor and cargo can interact either directly or facilitated by substrate-specific adaptor proteins (Calado et al., 2002). Transport receptors fall into two main categories, importins and exportins, based on their movement of cargo with an NLS or NES into or out of the nucleus (Grespin et al., 2008).

Characteristics of the karyopherin β family include a RanGTPase-binding motif, direct binding with nucleoporins, and cargo recognition in a Ran-dependent manner (Brownawell and Macara, 2002). The asymmetry of RanGTP binding to importins and exportins aids in the compartmentalization of cargo and the directionality of transport. In nuclear import, RanGTP facilitates disassociation of importins and cargo in the nucleus, while in nuclear export, RanGTP facilitates binding of exportins to their cargo (Brownawell and Macara, 2002). The complex moves through the NPC, RanGTP is converted to RanGDP by RanGTPase activating protein (RanGAP), and the protein complex dissociates (Calado et al., 2002).

IMPORT

The classical mechanism of nuclear import involves a heterodimer of importin α and importin β . Importin α binds to a protein containing a classical NLS (c-NLS). Then importin β binds to importin α and mediates transport of the trimer through the NPC (Kakuk et al., 2008). Once inside the nucleus, the complex disassociates when Ran-GTP binds to importin β . Then an exportin (CAS) bound to Ran-GTP transports importin α back out of the nucleus. Hydrolysis of Ran-GTP into Ran-GDP causes CAS to disassociate from the complex, leaving CAS in the cytoplasm, and the Ran-GDP is recycled to the nucleus (Goldfarb et al., 2004). Besides the

classical importin α/β pathway, there are pathways that use other members of the karyopherin β family. These karyopherins can bind to a variety of NLSs. Some NLSs contain basic residues, as seen in core histones, and are rich in arginines and glycines, like RNA-binding proteins. For other NLSs with relatively large domains that lack basic residues, the three dimension protein structure seems critical (Pemberton and Paschal, 2005). A wide variety of cargo can be transported into the nucleus, including NRs, through the importin α/β pathway (Kumar et al., 2006). Similarly, NRs can also use a variety of importins. For example, the glucocorticoid receptor can use its NLS-1 to be transported by the importin α/β , importin 7, or importin 8 pathways (Pemberton and Paschal, 2005; Kumar et al., 2006). Furthermore, we have shown recently that TR can use importin 7 or the importin $\alpha 3/\beta 1$ pathways to be transported from the cytoplasm into the nucleus (Parente, 2010) (Figure 1A).

EXPORT

The other side of the balancing act between transport into and out of the nucleus, is nuclear export of NRs by the karyopherin β superfamily. Most of the functions of the approximately 22 identified karyopherin β family members remain unknown. Nevertheless, two of the best characterized receptors are Exportin-t and CRM1. Exportin-t facilitates transport of tRNAs out of the nucleus by recognizing correct tertiary structure. Exportin-t selects for mature over premature tRNAs, but has no preference for specific tRNAs (Calado et al., 2002). CRM1 is a general transport receptor, meaning that it can export many different proteins. Cargo binds to a leucine-rich NES on the cargo to facilitate transport (Brownawell and Macara, 2002). Leptomycin B is a known specific inhibitor of CRM1 that has greatly facilitated studies of this exportin and helped reveal CRM1 independent export pathways (Calado et al., 2002) (Figure 1B). Exportin 5 (XPO5) is transport receptor that has been characterized based on its role in

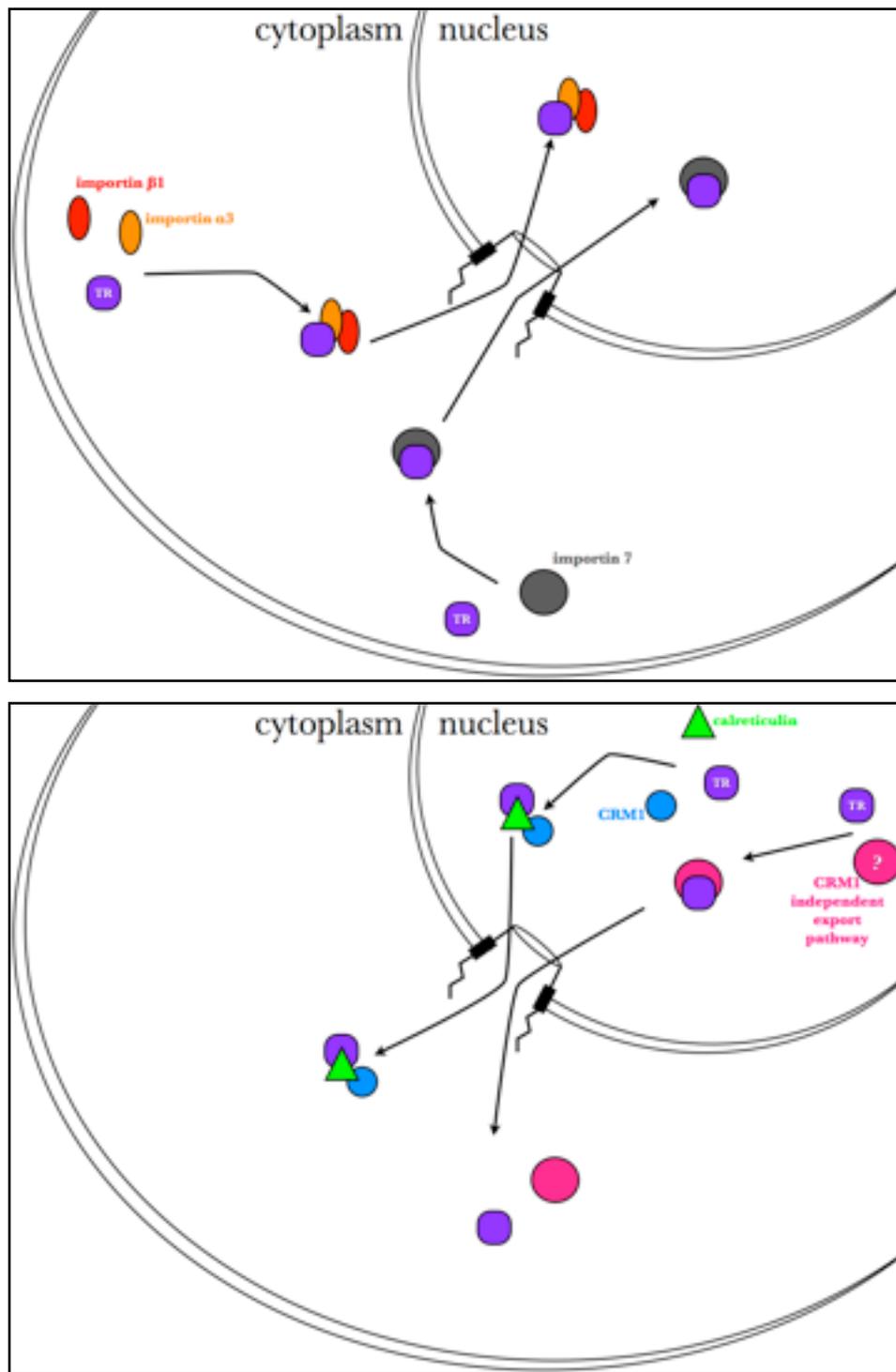


Figure 1: TR nuclear import and export

A. Left is classical import model, where importin $\alpha 3$ is used as an adaptor protein with importin $\beta 1$ to mediate nuclear import of TR. Right is an alternative pathway where nuclear import of TR is mediated by importin 7. B. Left is classical export model, where calreticulin binds to TR and CRM1 to mediate export out of the nucleus. Right represents CRM1 independent pathways, which have yet to be characterized for TR.

microRNA (miRNA) export. Another recently discovered protein that is thought to have transport receptor characteristics is the Ran Binding Protein 17 (RanBP17). These two proteins (XPO5 and RanBP17) will be the focus of this thesis.

The Discovery of XPO5

XPO5 was found during a database screen using the CRM1 sequence to identify other general export receptors (Brownawell and Macara, 2002). Through sequence analysis, a human clone (KIAA1291) was discovered with an incomplete open reading frame. Using the clone, 5' primers were designed to use in 5' rapid amplification of cDNA ends (RACE) to create a complete open reading frame (Brownawell and Macara, 2002). The resulting mature protein was named XPO5, because it was later shown to transport protein cargo from the nucleus to the cytoplasm, and it was fifth member of the mammalian karyopherin β family to be characterized (Brownawell and Macara, 2002).

Initially to see if XPO5 was a transport receptor, XPO5 was tested for its ability to bind to RanGTP and nucleoporins. A yeast two-hybrid assay showed that XPO5 could interact with Ran, specifically the NH₂-terminal region of XPO5 (Brownawell and Macara, 2002). Studies showing a direct interaction *in vitro* between Ran-GTP and XPO5 using GST-tagged Ran confirmed these results (Brownawell and Macara, 2002). XPO5 can also associate with the cytosolic nucleoporin Can/Nup214 and NPC basket nucleoporin Nup153, which confirmed that XPO5 is an exportin of the karyopherin β family (Brownawell and Macara, 2002). This association of XPO5 with nucleoporins is independent of RanGTP as seen in co-immunoprecipitation assays with XPO5 and RanQ69L, a Ran mutant locked in the GTP-bound form (Brownawell and Macara, 2002). Subsequent experiments revealed that XPO5 is conserved across plant, animal, and fungal kingdoms except for nematodes (Murphy et al., 2008). Another

set of investigations concluded that even though XPO5 is conserved from budding yeast to humans, there are some differences in cargo preferences (Shibata et al., 2006).

Cargo of the general transport receptor XPO5

XPO5 was first shown to export tRNA, and tRNA bound to eukaryotic elongation factor 1A (eEF1A), in a RanGTP-dependent manner (Calado et al., 2002). Through microinjection experiments, it was found that eEF1A localized in the cytoplasm when XPO5 was added (Calado et al., 2002). These assays involved translating eEF1A in the presence of ³⁵S-methionine *in vitro* in rabbit reticulocyte lysate then injecting it into the nuclei of *Xenopus laevis* oocytes (Calado et al., 2002). Subsequent studies confirmed that XPO5 facilitates transport of interleukin enhancer binding factor (ILF). Brownawell and Macara (2002) created mutant versions of ILF and tested their ability to bind to XPO5 using a ³²P-labeled dsRNA probe. It was concluded that transport depended on the double-stranded RNA binding domain (dsRBD) of XPO5 (Brownawell and Macara, 2002). Subsequent analysis of the dsRBD showed that XPO5 bound to its cargo's dsRBD in a strictly sequence independent manner (Bohnsack et al., 2004).

Further analysis of RanGTP competition experiments led to the discovery that microRNAs (miRNAs) are a cargo of XPO5 (Lund et al., 2004). miRNAs are part of a conserved system of RNA-based gene regulation at the post-transcriptional level. miRNAs are first made as imperfect RNA hairpins (pri-miRNAs) in the nucleus that are spliced by the RNase III Drosha into 60-80 nucleotide hairpin structures called pre-miRNAs (Yi et al., 2003; Bohnsack et al., 2004), and then exported to the cytoplasm. These pre-miRNAs are processed into short (~22 nucleotides) double stranded miRNAs by the RNase Dicer, and are then incorporated into the RNA-induced silencing complex (RISC). Through complementary base pairing the miRNAs lead the RISC to mRNAs targeted for degradation or translational repression (Yi et al., 2003).

XPO5 was also tested for direct interaction with ^{32}P -pre-miRNA to determine if XPO5 can bind pre-miRNAs, because previous to discovering XPO5, the export pathway of pre-miRNAs was unknown (Lund et al., 2004). As shown in short interfering RNAs (siRNAs) and RNA interference (RNAi) studies, XPO5 can interact directly with pre-miRNAs and is a key player in miRNA biogenesis (Lund et al., 2004; Leisegang et al., 2012). XPO5 not only acts as a nuclear export factor for miRNA, it also inhibits degradation of bound pre-miRNA by nucleases (Okada et al., 2009). Furthermore, when XPO5 expression is inhibited through siRNA, pre-miRNA is unable to suppress target mRNA expression. Thus, pre-miRNA export is dependent on XPO5 (Yi et al., 2003).

Structure of XPO5

The structure of XPO5 was determined by X-ray crystallographic analysis of XPO5 and signal information from crystals containing pre-miRNA 5-bromo-oxyuracil derivatives (Okada et al., 2009). Okada et al. (2009) modeled 1,082 XPO5 residues to ultimately find that the XPO5, RanGTP, and pre-miRNA complex is an ellipsoid with dimensions of 65Å by 80Å, by 110Å. XPO5 forms a baseball mitt-like structure, with a tunnel-like structure the cargo can pack (Okada et al., 2009). The export motif of pre-miRNA that is recognized by XPO5 is called a minihelix, which consists of a double stranded stem of at least 14 nucleotides long. The 5' end of the pre-miRNA is base paired, and the 3' end has a short unpaired section of nucleotides, which can tolerate imperfect base pairing and bends (Gwizdek et al., 2004). When pre-miRNA is the cargo of XPO5, the free two-nucleotide 3' end is inserted into the base of the tunnel and interacts with HEAT repeats 12-15 of XPO5. The 3' overhang is stabilized by forming salt bridges and hydrogen bonds with the amino acids of XPO5 (Okada et al., 2009). Structural studies also showed that binding of the pre-miRNA to XPO5 is a sequence-independent process,

and only depends on the length of the double-stranded stem and the two-nucleotide long 3' overhang (Okada et al., 2009).

RanBP17

Ran binding protein 17 (RanBP17) was first discovered in 2000 when Koch et al. were investigating a common break site and translocation area on chromosome eight, which is involved with acute lymphoblastic leukemia. Parts of the RanBP17 gene were isolated and sequenced, and Koch et al.'s results showed that RanBP17 shared 100% homology with the KIAA0745 gene, except for a 540bp insertion at position ~800 and a few differences in the 5' end region. Even though the initial experiments were done with murine genes, they showed a greater than 67% homology to the human gene equivalent (Lee et al., 2010). Structural analysis revealed that RanBP17 is a member of the importin β superfamily, because it has an importin β N-terminal domain (Koch, et al., 2000). RanBP17 and exportin 7 (also called RanBP16) are evolutionarily distinct from the rest of the NRs in the importin β superfamily. RanBP17 is thought to have evolved later, since it has only been seen in vertebrates (Kutay, 2000). In comparison to the importin β superfamily as a whole, RanBP17 has the highest sequence homology to CRM1. The characteristic importin β N-terminal domain is a small section within the larger CRM1 homology (Lee et al., 2010). Even though the cargo of RanBP17 has yet to be identified, with this sequence homology, it has been hypothesized to act like a nuclear transport receptor (Kutay, 2000).

LINK TO DISEASE:

Thyroid disease is one of the leading endocrine diseases worldwide, affecting almost a half billion people (Oetting and Yen, 2007). Serious risk for thyroid disease is caused by improper maintenance and function of thyroid hormones (T3 and T4), which regulate large

areas of gene expression through TRs. Localization of NRs play a key role in maintaining the normal function of cells. Mutations or post translational changes in NRs can cause excessive growth and cancer, especially in organs that have high NR activity like breast, ovary, pancreas, intestine, prostate, and lung (O'Malley and Kumar, 2009). For example when AR is mutated, it mislocalizes causing another steroid receptor (coactivator 1) to mislocalize, which has been linked to prostate cancer (Nazareth et al., 1999). Mutations and mislocalization of TR can lead to resistance to thyroid hormone, metabolic abnormalities, and thyroid cancer (Cheng et al., 2010).

Specific to TR, certain isoforms can also alter subcellular localization, such as v-ErbA, the oncogenic version of TR, found in chicken erythroblasts associated with the avian erythroblastosis virus (Oetting and Yen, 2007). v-ErbA dimerizes with RXR to change the subcellular localization of TR α (Bonamy et al., 2005). Mutations in TR β also show deleterious effects. To date, 124 different mutations have been found in TR β , with one well-characterized (Pro453His mutation), which were described to cause resistance to thyroid hormone (RTH) (Cheng et al., 2010). While most patients are heterozygotes for the TR β mutation with mild cases of RTH, there was one case of homozygous mutated TR β , where the individual displayed a severe RTH phenotype and died young (Ono et al., 1991).

Exportins have also been shown to be involved with disease. While little information is known about RanBP17, recent studies of miRNA biogenesis and XPO5 have led to discoveries linking XPO5 misregulation to tumorigenesis and disease. For example, adenovirus VA1 has been shown to use the XPO5 export pathway. VA1 can competitively inhibit other XPO5 cargo by saturating XPO5 for export. This saturation of XPO5 leads to the inability to export miRNAs and alters post-transcriptional gene regulation by miRNAs (Perron and Provost, 2009). Even though there is no direct link between cancer and XPO5, if miRNA levels are lowered by

expression of XPO5, then the individual's risk for developing cancer increases. Through genetic screening and epigenetic association studies of patients with variants of XPO5 and breast cancer, researchers found a link between changes in miRNA expression based on XPO5 levels and tumorigenesis (Leaderer et al., 2011). In another study, XPO5 was also shown to indirectly modulate apoptosis in cells, because XPO5 was able to export Fas-associated death domain protein (FADD) to the cytoplasm (Screaton et al., 2003).

Down regulation of miRNAs has been seen in many malignancies (ovarian, breast, and prostate), which suggests that miRNAs may be able to suppress tumorigenesis (Melo and Esteller, 2011). Frameshift mutations in exon 32 were found in the XPO5 gene in two MSI colon cancer cell lines and primary tumors. The mutant sequence encodes a truncated form of XPO5, which makes it unable to associate with pre-miRNA and export it into the cytoplasm (Melo and Esteller, 2011). Even in cells heterozygous for this mutation, there is less pre-miRNA exported and processed. This leads to less mature miRNA in the cytoplasm, and a greater chance of tumorigenesis. To provide further evidence for this conclusion, Melo and Esteller (2011) were able to rescue pre-miRNA export and processing by restoring XPO5 protein levels in the cells already producing the truncated form of XPO5. These rescued cells then showed tumor suppressing features.

THESIS OBJECTIVE:

Since TR mediates the cellular response to T3 and T4, TR is extremely important in development and disease. To maintain correct levels of TR inside the nucleus, nuclear export of TR is also critical for homeostasis. Mislocalization of nuclear receptors to the cytoplasm is related to thyroid diseases and cancer. Additionally, mutated TRs are involved in thyroid metabolism, and promotion of oncogenetic activities as seen through mislocalization of TR by v-

ErbA, lung carcinomas, some skin carcinomas, lymphomas, lymphocytic (Bcl3) or acute myeloid leukemias, (Bonamy et al., 2005; O'Malley and Kumar, 2009). When TR is exported from the nucleus, TR cannot bind to DNA, and therefore TR cannot regulate gene expression. Currently there are both CRM1-dependent and CRM1-independent export pathways that facilitate transport of TR from the nucleus to the cytoplasm (Grespin et al., 2008; Mavinakere et al., 2012). By characterizing CRM1-independent export pathways, like XPO5 and RanBP17, further insight into T3-responsive gene transcription and regulation will be attained. These findings may have further implications on the regulation of other members of the nuclear receptor superfamily as well.

The overall objective of this thesis research was to examine whether exportin 5 or RanBP17 are used in the nuclear export of TR, through over-expression of exportin 5 or RanBP17 in transfected cultured HeLa cells. Prior fluorescence recovery after photobleaching (FRAP) assays in our lab provide preliminary evidence that exportin 5 mediates the CRM1-independent nuclear export pathway for TR (Subramanian, 2012). In addition, we recently characterized a NES in helix 12 and up to two CRM1-independent NESs in the ligand-binding domain between helix 3 and helix 6 (Mavinakere et al., 2012). Thus, to determine which CRM1-independent NES in TR is recognized by exportin 5, TR constructs, which only have one of the two CRM1-independent NESs were analyzed. Even though little is known about the cargo of RanBP17, it was hypothesized that RanBP17 can also mediate TR export into the cytoplasm, based on sequence homology with CRM1.

METHODS

PLASMIDS

The plasmid pGFP-TR α 1 encodes green fluorescent protein (GFP)-tagged, full-length rat TR α 1, while pGFP-TR β 1 encodes the full length GFP-tagged human TR β 1 (Mavinakere et al., 2012). The nuclear export sequence expression plasmids encoding GFP-GST-GFP-Hinge-LBD Δ 269-410 (NES-H3/H6) and GFP-GST-GFP-NES-H12-Hinge mutant TR α 1 plasmids were described previously (Mavinakere et al., 2012).

The plasmid pKmyc-Exp5 encodes a Myc-tagged functional exportin-5 protein and was acquired from Addgene (Addgene #12552) (Brownawell and Macara, 2002). The HA-tagged RanBP17 expression plasmid was a gift from C. Smas (University of Toledo College of Medicine, Ohio) pCMV-Myc (#631604) was acquired from Clontech, while pCMV-HA (#K6003-1) was obtained from BD Biosciences. The pCAT $\text{\textcircled{3}}$ -Basic Vector (#E1871) was acquired from Promega, while the tk-TREp-CAT plasmid was a gift from R. Evans (Salk Institute for Biological Studies). The tk-TREp-CAT plasmid is a TRE linked with the herpes simplex virus thymidine kinase (tk)-chloramphenicol acetyltransferase (CAT) fusion gene reporter. These plasmids were subcloned into DH5 α cells, allowed to amplify, then purified with a Qiagen midi-prep kit using the manufacturer's protocol. The amount of DNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies).

CELL CULTURE

HeLa cells were cultured under Biosafety level II conditions using a Labconco cabinet. HeLa cells were grown at 37°C under 5% CO $_2$ and 98% humidity in a ThermoScientific incubator to 70-90% confluency. Cells were cultured in filter-capped flasks (Nunc) in Minimum Essential Medium (Gibco), enhanced with 10% fetal bovine serum (FBS) (Invitrogen). The

antibiotics penicillin (100 units/mL) and streptomycin (100µg/mL) were intermittently added to the growth medium.

To seed 6 well plates with glass coverslips, HeLa cells were first washed with D-PBS (D-PBS: 0.10g KCL, 0.10g KH₂PO₄, 4.00g NaCl, and 1.08g Na₂HPO₄·7H₂O in 500 mL of H₂O). Cells were dislodged from the flask by incubating them with 0.25% trypsin for 2 minutes at room temperature, aspirating off the trypsin, and then incubated at 37°C for a subsequent 2 minutes. Cells were then resuspended in Minimum Essential Medium with FBS, counted using a hemocytometer, and seeded at 2.5-3.0 x 10⁵ cells per well in 2mL of medium on a coverslip (Fisher) in a 6-well plate (Costar). The HeLa cells were incubated at 37°C for approximately 24 hours, until the cells reached about 70% confluency.

TRANSIENT TRANSFECTION

Expression plasmids were transfected into HeLa cells to visualize the subcellular localization of the fluorescently-tagged proteins by fluorescence microscopy. Cotransfections of two or more plasmids were carried out to introduce and simultaneously overexpress multiple proteins within the cells. For transient transfections, 2µg of total plasmid DNA was diluted in 250µL of Opti-MEM I Reduced Serum Medium (Invitrogen). For cotransfections, the 2µg of plasmid DNA was split between the two plasmid types. For example, when transfecting with TRα1 and pKmyc-Exp5, 0.5µg of TRα1 and 1.5µg of pKmyc-Exp5 were added. A second solution (250µL total) was made of 4µL of Lipofectamine 2000 Reagent (Invitrogen) in 246µL Opti-MEM I Reduced Serum Medium. The two mixtures were incubated separately for 5 minutes, before they were combined and incubated for 20 minutes. Then, 500µL of the combined solution was added to each seeded coverslip in the 6-well plate. After a 7 to 8 hour

incubation at 37°C, the Opti-MEM media was replaced with 2 mL of Minimum Essential Medium with FBS per well.

FIXATION AND IMMUNOFLUORESCENCE

Approximately 24 hours post transfection, the HeLa cells were fixed and stained for visualization and analysis by fluorescence microscopy. The Minimum Essential Medium with FBS was removed, and the cells were washed three times with 2mL of D-PBS for 15 seconds. The cells were fixed with a 3.7% formaldehyde solution in D-PBS for 10 minutes in a fume hood (Fisher Hamilton). After the formaldehyde was disposed of in the designated waste container, the cells were washed with 2mL D-PBS three more times for 5 mins. Cells were permeabilized with 0.2% Triton-X in D-PBS solution for 5 minutes, then washed three more times with 2mL D-PBS three times per well for 5 minutes. The coverslips were inverted on 30µL of an antibody mix of 1:500 dilution of primary antibody, 1.5% normal goat serum (#PCN5000 Invitrogen), and D-PBS. The primary antibody used for XPO5 was an anti-Myc antibody (c-Myc mouse monoclonal antibody, Clontech, Cat#631206, Lot#1104389A) and for the HA-tagged proteins an anti-HA antibody was used (rabbit polyclonal antibody to HA tag, Abcam #ab9110, Lot# GR20600-3). The coverslips were flipped using tweezers onto parafilm, which was suspended in a humidified storage chamber for 1.5 hours. The humidified chamber consisted of parafilm suspended with tape above a damp paper towel. At the end of the incubation, the coverslips were flipped back into the 6-well plates face up, using tweezers. After washing the wells three times with 2mL D-PBS for 5 minutes, the coverslips were inverted, using tweezers, on 30µL of an antibody mix of 1:500 dilution of secondary antibody, 1.5% normal goat serum (#PCN5000 Invitrogen), and D-PBS. The secondary antibody for Myc-tagged protein assays was CY3 conjugated Goat Anti-Mouse IgG(H+L) (ZyMax, Invitrogen #81-6515, Lot: 382943A), while

Texas Red Anti-Rabbit IgG(H+L) (Vector Laboratories, TI-1000, Lot: S0504) was used for HA-tagged protein assays. The coverslips were incubated for 45 minutes in a humidified chamber in the dark. Next, the coverslips were flipped back into the 6-well plate, and washed again three times with D-PBS for 5 mins each time. After draining the excess liquid by touching the edge of the coverslip to a KimWipe (Kimberly-Clark), the coverslips were mounted using GelMount with DAPI (2 μ L of 0.25 μ g/ μ L DAPI stock per 1 mL of GelMount) onto glass slides (Fisherfinest, Fisher Scientific), using tweezers. The slides were stored flat in a slide box at 4°C for approximately 24 hours before scoring.

FLUORESCENCE MICROSCOPY

The mounted slides were analyzed with the Nikon Eclipse TE2000E fluorescence microscope (Sigma, Melville, NY) using the inverted 60x oil immersion objective. Three filters were used to determine subcellular localization patterns. The UV-2E/C light filter block was used to visualize the DAPI-stained nuclei. The FITC (blue light) B-2E/C filter block excited the GFP-tagged TR α 1 and TR β 1 proteins, which fluoresced green. The TRITC (green light) T-2E/C filter block allowed visualization of the fluorophore-conjugated secondary antibodies (anti-Myc, anti-HA), which emit red fluorescence. Pictures of the cells were taken using a Photometrics Cool Snap HQ₂ camera or a Nikon A1Rsi microscope Ti-E-PFS camera, with a 40x water objective. NIS-Elements 2.30 software and Adobe Photoshop CS were used to normalize and layer images. Adobe Illustrator was used to construct figures, and Mac OS X Numbers was used to construct charts.

CELL SCORING WITH ANALYSIS

Before cells were scored, they were blinded by another lab member to control for a scoring bias. At least three trials of three slides each, with a minimum of 100 cells scored per

slide, were conducted for each experimental group. Transfections of pGFP-TR α 1 or pGFP-TR β 1 with either pCMV-Myc, pmyc-Exp5, pCMV-HA, or pRanBP17 plasmids were scored into two categories: primarily nuclear, or nuclear and cytoplasmic. Transfections with TR α 1 NES expression plasmids, GFP-GST-GFP-Hinge-LBD Δ 269-410(NES-H3/H6) and GFP-GST-GFP-NES-H12-Hinge, and either pCMV-Myc or pmyc-Exp5, were categorized into four groups: nuclear, nuclear greater than cytoplasmic, whole cell, and nuclear less than cytoplasmic. After the counts were complete, the slides were unblinded and statistical tests were performed. Statistical tests included averages, standard deviation, and standard error.

CAT ELISA

Chloramphenicol Acetyltransferase (CAT) Enzyme-linked Immunosorbent Assay (ELISA) was performed to quantify TR-mediated transactivation of a CAT reporter gene, under control of a TRE. The CAT protein is only made in the presence of TR, so the amount of CAT protein produced correlates with the amount of TR in the nucleus. HeLa cells were seeded at approximately 7.0×10^5 cells per 100mm dish with 10mL of Minimum Essential Medium with FBS. After incubating for approximately 24 hours at 37°C, the cells were transfected with a total of 10 μ g of plasmid DNA diluted in 1.25mL of Opti-MEM I Reduced Serum Medium (Invitrogen). Since these were cotransfections, the 10 μ g of plasmid DNA was split between the plasmid types. The pCAT@3-Basic Vector with pGFP-TR α 1 control had 5 μ g for each plasmid, while each experimental group had 3 plasmids with 3.33 μ g of each plasmid. For example, when transfecting with TR α 1 and pKmyc-Exp5 (XPO5 expression plasmid), 3.33 μ g of pGFP-TR α 1, 3.33 μ g of ptk-TREp-CAT, and 3.33 μ g of pKmyc-Exp5 were added. A second solution (1.25mL total) was made of 20 μ L of Lipofectamine 2000 Reagent (Invitrogen) in 1.23mL Opti-MEM I Reduced Serum Medium. The two mixtures were incubated separately for 5 minutes, before

they were combined and incubated for 20 minutes. Then, 2.5mL were added to each seeded 100mm dish. After a 6 hour incubation at 37°C, the Opti-MEM media was replaced with 10 mL of Minimum Essential Medium with FBS per well. Approximately 12-13 hours post-transfection, the medium was replaced again with Minimum Essential Medium containing 10% charcoal-dextran stripped FBS. Half of the 100mm plates (one of each experimental category) were supplemented with 110 nM T₃.

Approximately 24 hours post transfection, the media was aspirated off in the Labconco biosafety cabinet, then plates were washed carefully three times with 5mL of pre-cooled D-PBS. Next, the cells were prepared for ELISA according to the manufacturer's protocol (Roche Applied Sciences). In brief, cells were lysed by incubating them in 1.5mL lysis buffer with protease inhibitors on ice for 30mins. Subsequently, cells were scraped off the plates, lysates were centrifuged for 10 mins at 4°C to remove any cellular debris, and protein concentration was determined using the ND-1000 spectrophotometer (NanoDrop Technologies). The amount of protein was adjusted to 600µg per 500µL with lysis buffer. The CAT protein working dilution was also prepared fresh for each experiment by first diluting a CAT stock solution in sample buffer, then creating working dilutions of 0, 0.125, 0.25, 0.5, and 1.0 ng of CAT enzyme per milliliter.

For the ELISA assay, 200µL of either the blank (POD substrate), working CAT dilutions, or cell extracts were added to each well on the ELISA microplate, with two replicates per plate. The cell extracts were covered with foil and incubated at 37°C for 1 hour. Then the extracts were removed, the microplate was washed 5 times with 250µL washing buffer for 30 seconds each, and the 200µL Anti-CAT-Digoxigenin (DIG) working dilution was added to each well. After incubating covered with foil for 1 hour at 37°C, the Anti-CAT-DIG was removed, the

microplate wells were washed 5 times with 250 μ L of washing buffer for 30 seconds each, and 200 μ L of Anti-DIG-peroxidase (POD) working dilution was added to each well. After another 1 hour incubation covered in foil at 37°C, the Anti-DIG-POD was removed, the microplate was again washed 5 times with 250 μ L of washing buffer for 30 seconds each, and 200 μ L of POD substrate was added to each well. The POD substrate was incubated at room temperature (15-25°C) until a green color change was noted (approximately 15 minutes). The plate was gently tapped to ensure a homogenous distribution of the colored product in each well. The absorbance was measured at 405nm with a microplate reader (BioRad, Model 550), using the Mes#3, Ref#2 function. The absorbance readout was analyzed against the standard CAT enzyme working dilution curve using Mac OS X Numbers.

RESULTS

EXPORTIN 5 (XPO5) CAN MEDIATE TR NUCLEAR EXPORT

Prior experiments have shown that knockdown of XPO5 slows TR shuttling (Subramanian, 2012), suggesting the XPO5 plays an important role in mediating CRM1-independent nuclear export of TR. To confirm and extend these findings, we determined whether XPO5 overexpression would change the nucleocytoplasmic distribution of TR. We hypothesized that XPO5 overexpression would shift the localization of TR from the nucleus to the cytoplasm.

First, we carried out control experiments to establish the distribution patterns for GFP-TR α 1, GRP-TR β 1, Myc, and the HA tag. Expression plasmids were transfected into HeLa cells, and the distribution of the proteins was visualized by fluorescence microscopy, either directly for GFP-tagged proteins, or by indirect immunofluorescence with antibodies specific for Myc and HA (Figure 2). As expected, the distribution of both TR α 1 and TR β 1 was primarily localized to

the nucleus (Bunn et al., 2001; Subramanian, 2012), with TR β 1 having a slightly greater cytosolic distribution than TR α 1 (Baumann et al., 2001). The Myc and the HA tags showed a whole cell distribution.

Next, we transfected HeLa cells with expression plasmids for GFP-TR (α 1 or β 1), and either Myc-XPO5 or Myc as a control. To visualize Myc-XPO5, the cells were immunostained with anti-Myc antibodies. Cells that were successfully co-transfected were analyzed under the fluorescence microscope, and the TR distribution pattern was scored for a primarily nuclear, or nuclear and cytoplasmic distribution.

Consistent with our hypothesis, when XPO5 was overexpressed, TR shifted towards a significantly greater cytosolic distribution ($p < 0.001$). In the cells expressing GFP-TR α 1 and Myc (control), 90.1% ($\pm 1.3\%$) of cells showed a nuclear distribution TR α 1, and 9.9% ($\pm 1.3\%$) of cells showed a nuclear and cytosolic localization (Figure 3). When cells were expressing GFP-TR α 1 and Myc-XPO5, cells showed a 52.5% ($\pm 2.1\%$) nuclear distribution of TR α 1, and 48.5% ($\pm 2.1\%$) of cells had a nuclear and cytosolic localization (Figure 3). In the cells expressing GFP-TR β 1 and Myc (control), 76.1% ($\pm 1.6\%$) of cells had a nuclear distribution of TR β 1, and 23.9% ($\pm 1.6\%$) of cells had a nuclear and cytosolic localization (Figure 4). When cells were expressing GFP-TR β 1 and Myc-XPO5, 32.8% ($\pm 2.5\%$) of cells had a nuclear distribution of TR β 1, and 67.2% ($\pm 2.5\%$) of cells had a nuclear and cytosolic localization (Figure 4). These overexpression experiments show that the distribution of TR can change from the overexpression of an export pathway. In conclusion, for both TR α 1 and TR β 1, overexpression of XPO5 caused a significant increase in their cytosolic distribution, suggesting that XPO5 plays a key role in the export of TR.

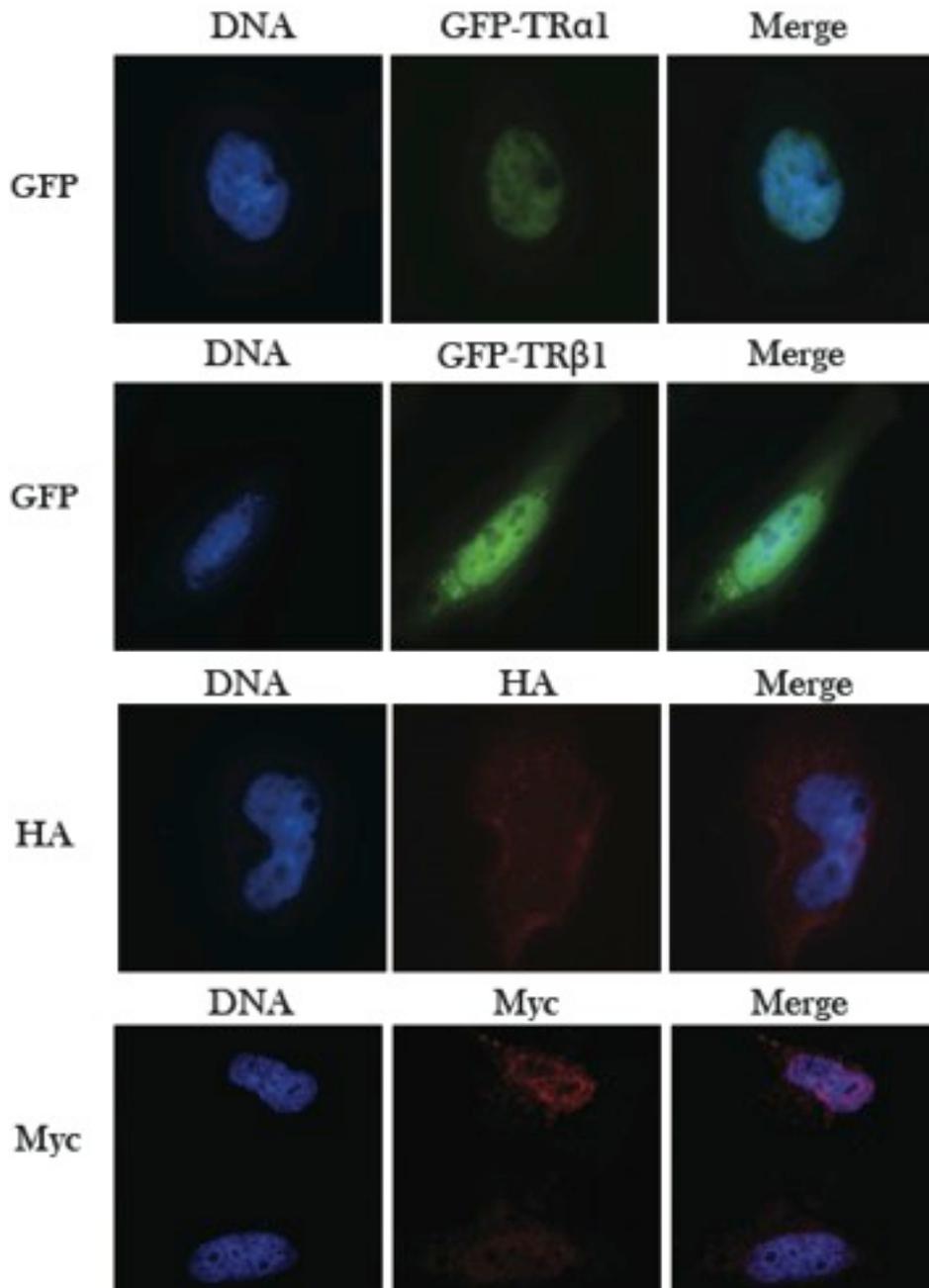


Figure 2: Subcellular distribution of GFP-TR α 1, GFP-TR β 1, Myc, or HA

HeLa cells were transiently transfected with GFP-TR α 1, GFP-TR β 1, Myc, or HA expression plasmids. After approximately 24 hours the HeLa cells were fixed. For visualizing Myc, cells were immunostained with anti-Myc primary and Goat Anti-Mouse IgG(H+L) secondary antibodies (red), before they were analyzed by fluorescence microscopy. For visualizing HA, cells were immunostained with anti-HA primary and Texas Red Anti-Rabbit IgG(H+L) secondary antibodies (red), before they were analyzed by fluorescence microscopy.

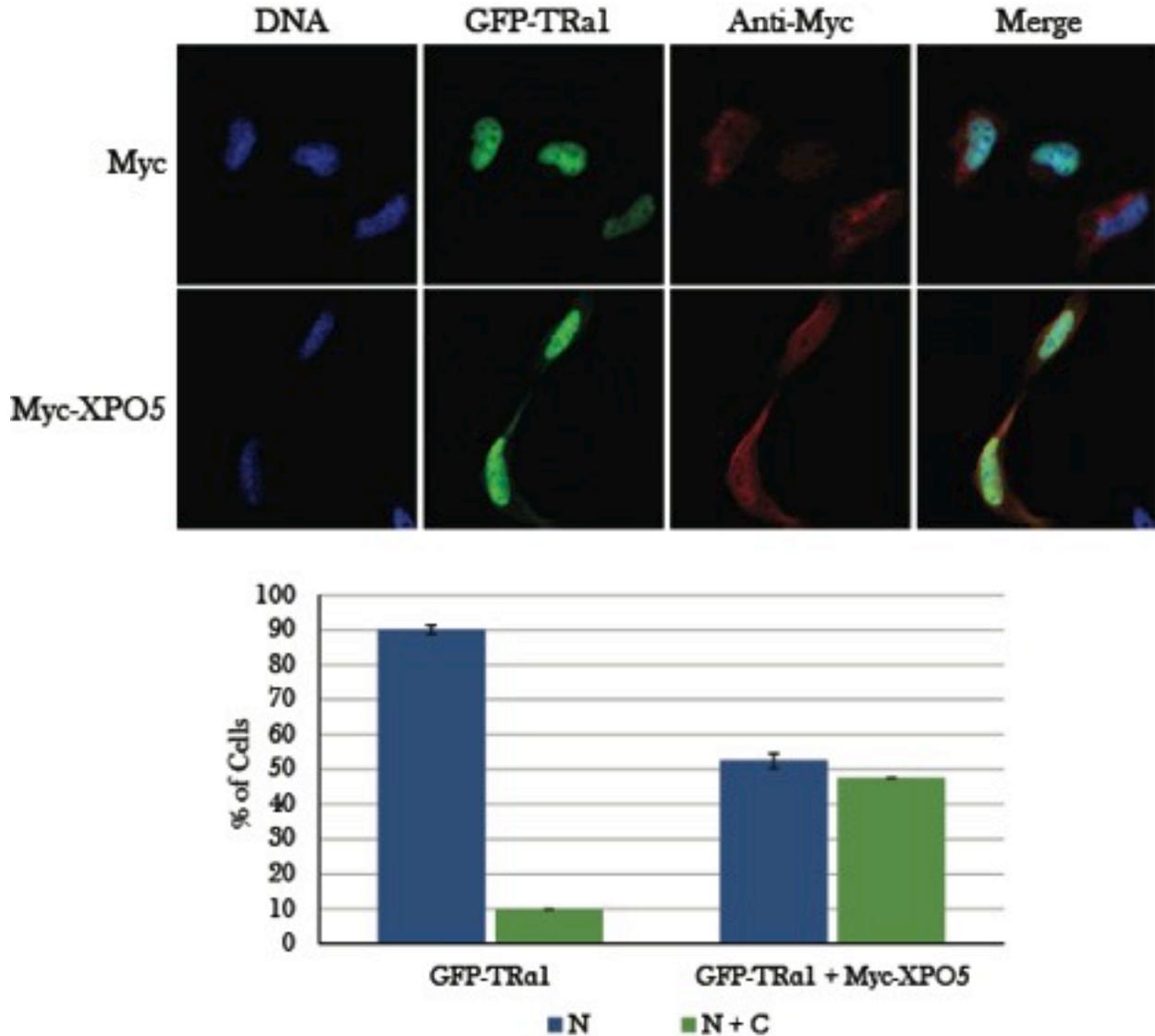


Figure 3: Overexpression of XPO5 increases nuclear export of TRα1

HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and either Myc or Myc-XPO5 expression plasmids. After approximately 24 hours the HeLa cells were fixed. The cells were then immunostained with anti-Myc primary and Goat Anti-Mouse IgG(H+L) secondary antibodies (red), before they were analyzed by fluorescence microscopy. GFP-TRα1 is visualized as green, while DAPI-stained nuclei are visualized as blue. The bar graph summarizes the distribution of GFP-TRα1 in HeLa cells, based on categories of primarily nuclear (N), or nuclear and cytoplasmic (N+C). Error bars are SEM, and the Chi-square test had a p-value < 0.001 with four replicate experiments of three slides each with at least 100 cells scored per slide.

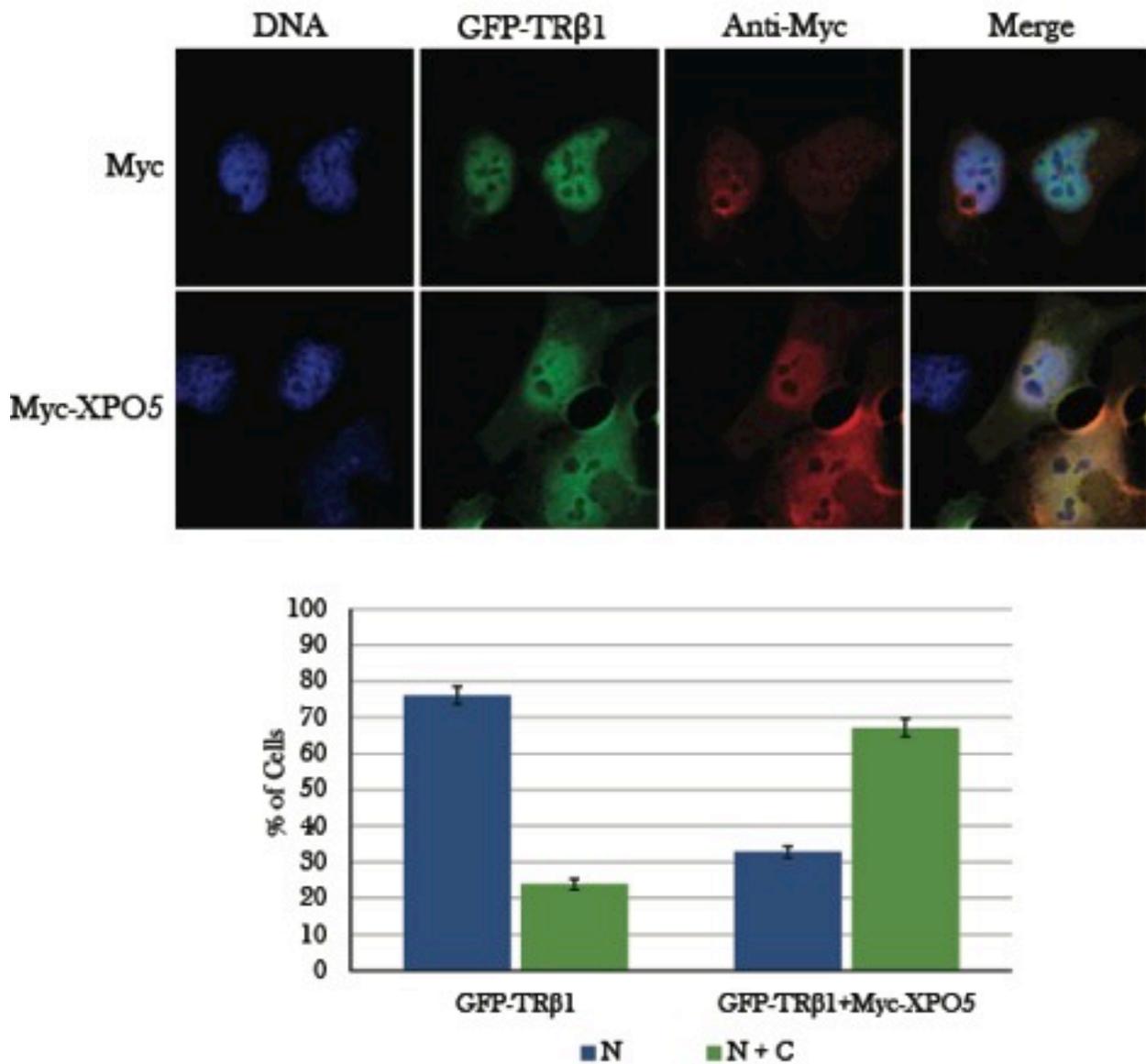


Figure 4: Overexpression of XPO5 increases nuclear export of TRβ1

Parallel experiments of the GFP-TRα1 were conducted with GFP-TRβ1 expression plasmid and either Myc or Myc-XPO5 expression plasmid. The bar graph summarizes the GFP-TRβ1 distribution of the HeLa cells from overexpressing Myc-XPO5 based on categories of primarily nuclear (N) or nuclear and cytoplasmic (N+C). Error bars are SEM and the Chi-square test had a p-value < 0.001 with four replicate experiments of three slides each with at least 100 cells scored per slide.

OVEREXPRESSION OF XPO5 DECREASES TR-MEDIATED CAT REPORTER GENE EXPRESSION

TR acts as a transcription factor for T₃ responsive genes. Since TR must be located within the nucleus to function as a transcription factor, we hypothesized that XPO5 overexpression would reduce TR-mediated gene expression. To further our understanding of XPO5 export, we used a CAT reporter gene under the positive control of a TRE to examine the transcriptional activity of GFP-TR (α 1 or β 1) (Figure 5). HeLa cells were transfected with expression plasmids for GFP-TR (α 1 or β 1), tk-TREpCAT reporter plasmid, and either Myc-XPO5, or Myc as a control. After 12 hour incubation with 100nM of T₃, whole cell lysates were used for CAT ELISAs to determine CAT protein levels. When XPO5 was overexpressed in the presence of T₃, the CAT enzyme levels were dramatically lowered. For TR α 1, the amount of CAT protein produced without XPO5 overexpression was four times greater than when XPO5 was overexpressed. Similarly, for TR β 1 the amount of CAT protein decreased by half when XPO5 was overexpressed. These CAT ELISA results support a model in which export of TR provides an important level of control for T₃ responsive gene expression.

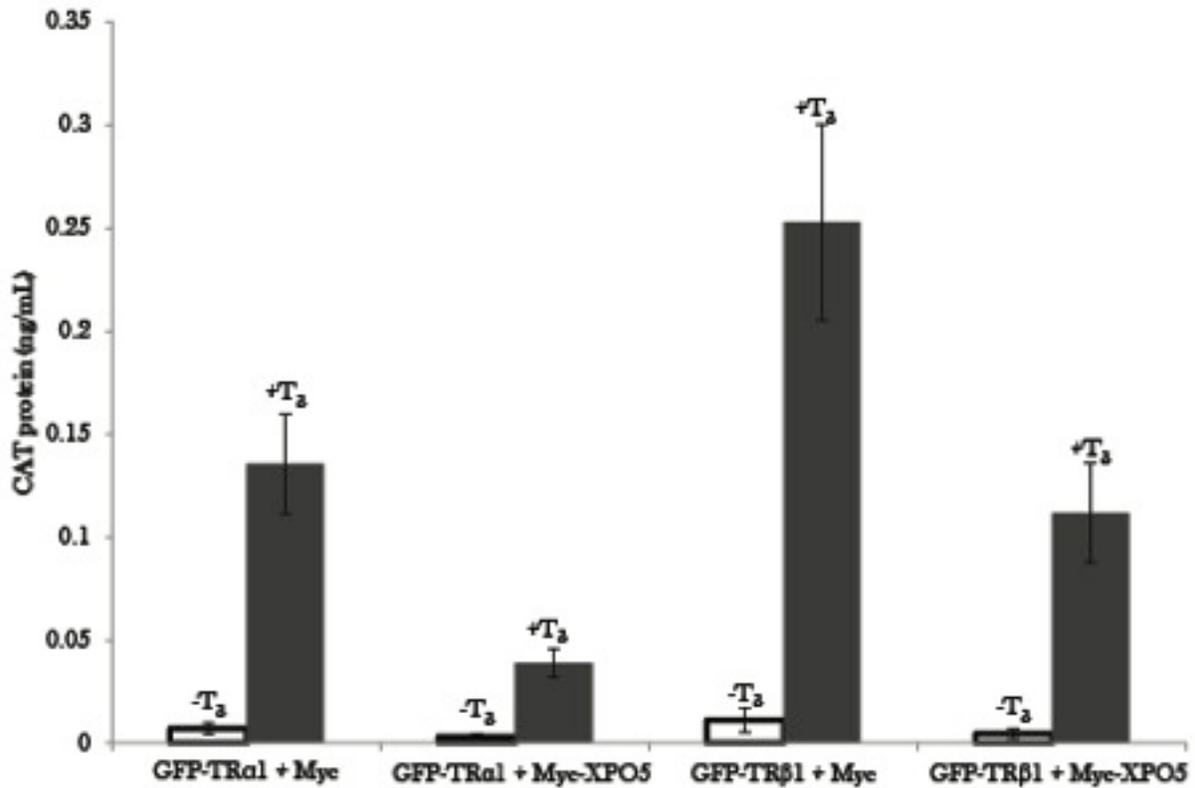


Figure 5: Overexpression of XPO5 decreases TR-mediated CAT reporter gene expression

Myc or Myc-XPO5 expression plasmids, GFP-TR(α 1 or β 1) expression plasmids, and tk-TREp-CAT reporter plasmids were overexpressed through transient transfection of HeLa cells. CAT ELISAs were conducted to measure CAT protein levels in whole cell extracts 12 hours after addition of 100nM T₃. Reporter gene expression (CAT protein) was measured in ng/mL (n=4). As a control, when GFP-TR (α 1 or β 1) expression plasmids were co-transfected with Myc-XPO5 in the absence of tk-TREp-CAT, there was no measurable CAT protein (data not shown). The error bars in the bar chart represent SEMs.

TR NUCLEAR EXPORT MEDIATED BY XPO5 REQUIRES NES-H12

After demonstrating a role for XPO5 in TR nuclear export, we next examined which NES in TR is required for this export pathway, where each mutant includes a NES and a NLS. The two expression plasmids used in these experiments were GFP-GST-GFP-Hinge-LBD(Δ 269-410), which includes the Hinge region NLS, and the NES motifs located in helix 3 and helix 6; and GFP-GST-GFP-Hinge-NESH12, which includes the Hinge NLS, and the NES located at helix 12 (Mavinakere et al., 2012). We hypothesized that the effect of XPO5 on TR export would be dependent on the presence of one or the other NESs. This hypothesis was tested using transient transfection assays in HeLa cells. Expression plasmids of either GFP-GST-GFP-Hinge-LBD(Δ 269-410) or GFP-GST-GFP-Hinge-NESH12 were co-transfected with either Myc-XPO5 or Myc. To visualize the Myc-tagged proteins, the cells were immunostained with anti-Myc antibodies. Cells that were successfully co-transfected were analyzed under the fluorescence microscope and the distribution of the TR NES constructs was scored. The NES constructs have an altered subcellular distribution compared with full length TRs; therefore, to reflect this difference constructs were scored into four categories: nuclear, nuclear greater than cytoplasmic, whole cell, and nuclear less than cytoplasmic.

Consistent with our hypothesis of the effect of XPO5 being NES-specific, when XPO5 was co-expressed with GFP-GST-GFP-Hinge-NESH12, there was a shift towards a more cytosolic distribution, whereas the distribution of GFP-GST-GFP-Hinge-LBD(Δ 269-410) with and without XPO5 was not statistically different ($p < 0.001$) (Figures 6-7). In the cells expressing GFP-GST-GFP-Hinge-LBD(Δ 269-410) and Myc (control), 40.1% ($\pm 3.9\%$) of cells had a whole distribution of the NES-H3/H6 construct, and 59.9% ($\pm 3.9\%$) of cells had a nuclear and cytosolic localization. When cells were expressing GFP-GST-GFP-Hinge-LBD(Δ 269-410) and Myc-XPO5, 0.8% ($\pm 0.41\%$) of cells had a nuclear greater than cytoplasmic distribution of the

NES-H3/H6 construct, 31.2% ($\pm 4.4\%$) of cells had a whole cell distribution, and 68.0% ($\pm 4.8\%$) of cells had a nuclear less than cytoplasmic localization (Figure 6).

When cells were expressing GFP-GST-GFP-Hinge-NESH12 and Myc (control), 24.7% ($\pm 1.7\%$) of cells showed a nuclear distribution of the NES-H12 construct, 28.8% ($\pm 2.8\%$) of cells had a nuclear greater than cytoplasmic distribution, 44.9% ($\pm 3.2\%$) of cells had a whole cell distribution, and 1.6% ($\pm 0.4\%$) of cells had a nuclear less than cytosolic localization (Figure 7). In striking contrast, in the cells expressing GFP-GST-GFP-Hinge-NESH12 and Myc-XPO5, only 6.9% ($\pm 2.2\%$) of cells had a nuclear distribution of the NES-H12 construct, 10.8% ($\pm 2.5\%$) of cells had nuclear greater than cytoplasmic distribution, 55.0% ($\pm 2.3\%$) of cells had a whole cell distribution, and 27.4% ($\pm 3.7\%$) of cells had nuclear less than cytosolic localization (Figure 7). This shift towards a more cytoplasmic distribution of the NES-H12 construct when XPO5 is overexpressed suggests that to mediate nuclear export of TR, XPO5 requires the NES located in helix 12.

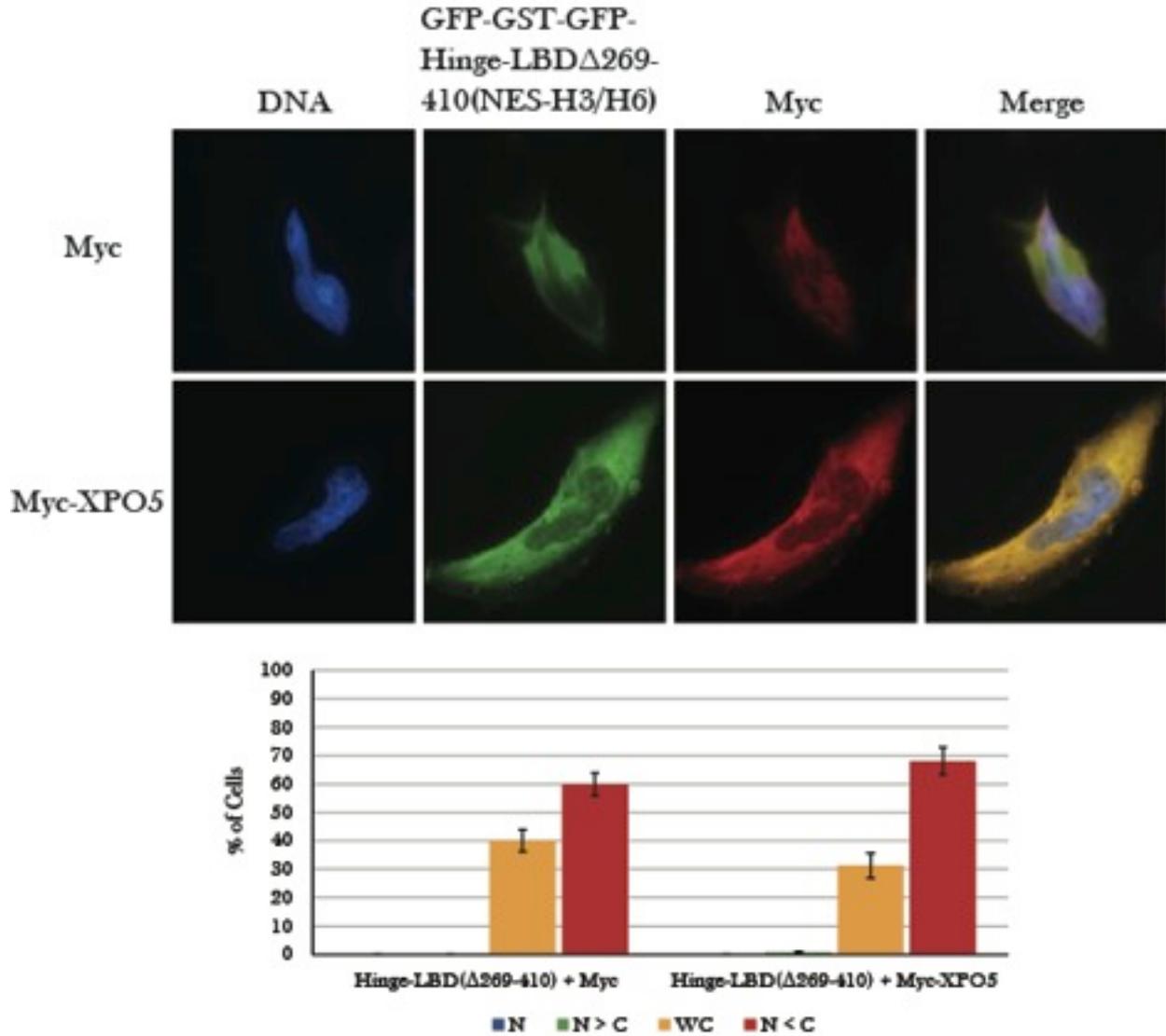


Figure 6: Overexpression of XPO5 does not promote nuclear export of TR via NES-H3/H6

Transient transfection experiments were conducted with GFP-GST-GFP-Hinge-LBD(Δ 269-410) expression plasmid and either Myc or Myc-XPO5 expression plasmids. The bar graph summarizes the distribution of the NES-H3/H6 construct in HeLa cells overexpressing Myc-XPO5 based on four categories: primarily nuclear (N), nuclear greater than cytoplasmic (N>C), whole cell (WC), or nuclear less than cytoplasmic (N<C). Error bars are SEM and Chi-square had a p-value < 0.001 with four replicate experiments of three slides each (100+ cells scored per slide).

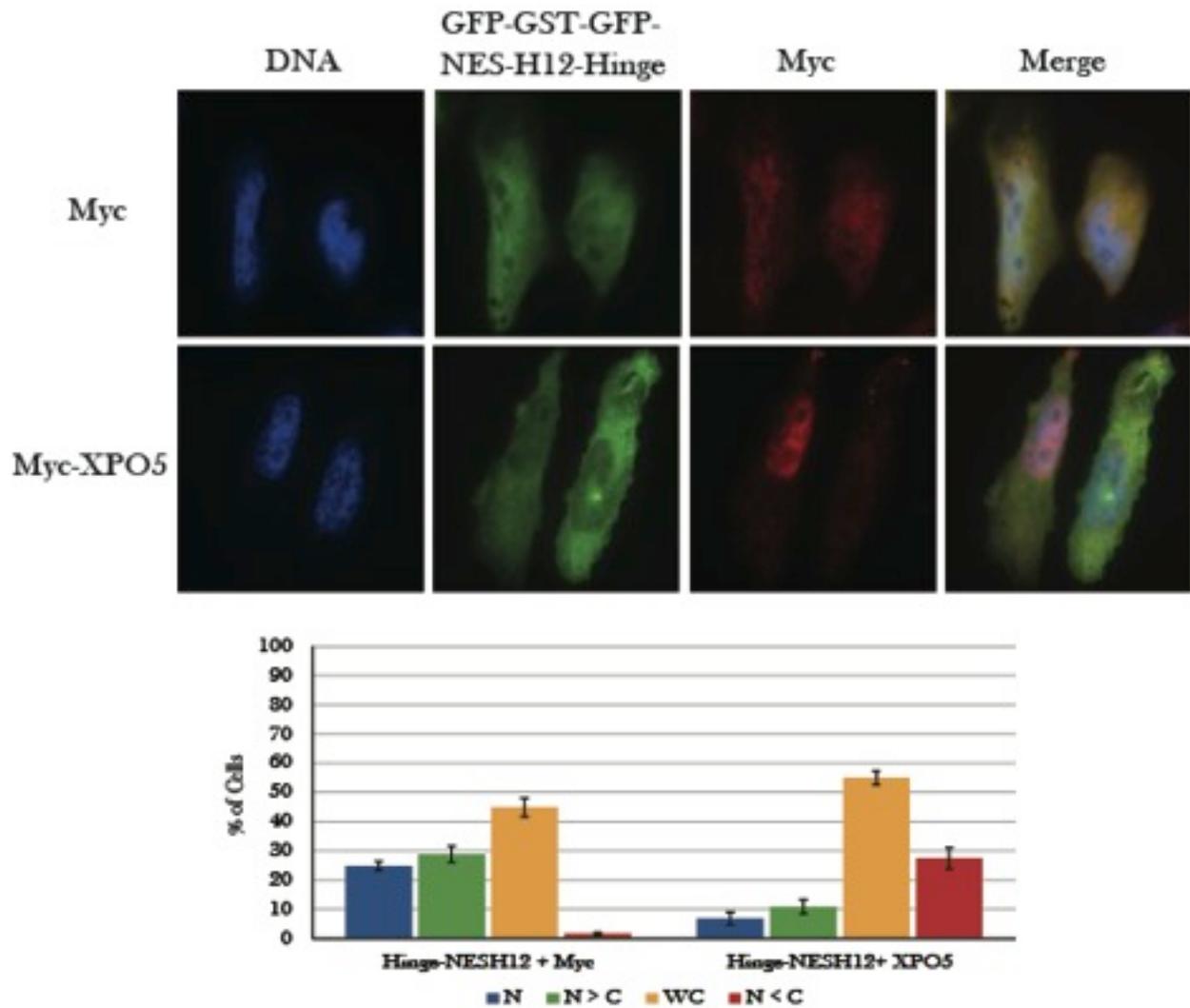


Figure 7: Overexpression of XPO5 promotes nuclear export of TR via NES-H12

Transient transfection experiments were conducted with GFP-GST-GFP-Hinge-NESH12 expression plasmid and either Myc or Myc-XPO5 expression plasmids. The bar graph summarizes the distribution of the NES-H12 construct in HeLa cells overexpressing Myc-XPO5 based on four categories: primarily nuclear (N), nuclear greater than cytoplasmic (N>C), whole cell (WC), or nuclear less than cytoplasmic (N<C). Error bars are SEM and Chi-square had a p-value < 0.001 with four replicate experiments of three slides each (100+ cells scored per slide).

RanBP17 CAN MEDIATE NUCLEAR EXPORT OF TR β 1 BUT NOT TR α 1

We examined the possible role of RanBP17 as another alternative export pathway for TR based on the homology between RanBP17 and CRM1 (Koch et al., 2000). We hypothesized that overexpression of RanBP17 would shift the localization of TR (α 1 or β 1) from the nucleus to the cytoplasm. This hypothesis was tested by transfecting HeLa cells with expression plasmids for GFP-TR (α 1 or β 1), and either HA-RanBP17 or HA as a control. To visualize the HA-tag, the cells were immunostained with anti-HA (primary) and Texas Red (secondary) antibodies. Cells that were successfully co-transfected were analyzed under the fluorescence microscope, and scored for a primarily nuclear, or nuclear and cytoplasmic distribution of TR.

Interestingly, RanBP17 overexpression did not promote nuclear export TR α 1, but significantly enhanced nuclear export of TR β 1 ($p < 0.001$) (Figures 8-9). Since less is known about TR β 1, it is possible that TR β 1 has different NES motifs compared with TR α 1. The HA-tag expression plasmid itself seemed to have some effect on TR distribution, thus controls of only TR α 1 or only TR β 1 were conducted as well. When only TR α 1 was transfected, 79.8% ($\pm 1.3\%$) of cells had a nuclear distribution of TR α 1, and 20.2% ($\pm 1.3\%$) of cells had a nuclear and cytosolic distribution. In the cells expressing GFP-TR α 1 and HA (control), there was a slight shift towards a more cytosolic distribution; 68.1% ($\pm 5.7\%$) of cells had a nuclear distribution of TR α 1, and 31.9% ($\pm 5.7\%$) of cells had a nuclear and cytosolic localization (Figure 8). When cells were co-expressing GFP-TR α 1 and HA-RanBP17, the distribution was comparable to the HA tag-transfected cells; 65.4% ($\pm 4.4\%$) of cells had a nuclear distribution of TR α 1, and 34.6% ($\pm 4.4\%$) of cells had a nuclear and cytosolic localization (Figure 8).

In TR β 1-only transfections, 64.0% ($\pm 4.7\%$) of cells had a nuclear distribution of TR β 1, and 36.0% ($\pm 4.7\%$) of cells had a cytosolic distribution (Figure 9). In the cells expressing GFP-

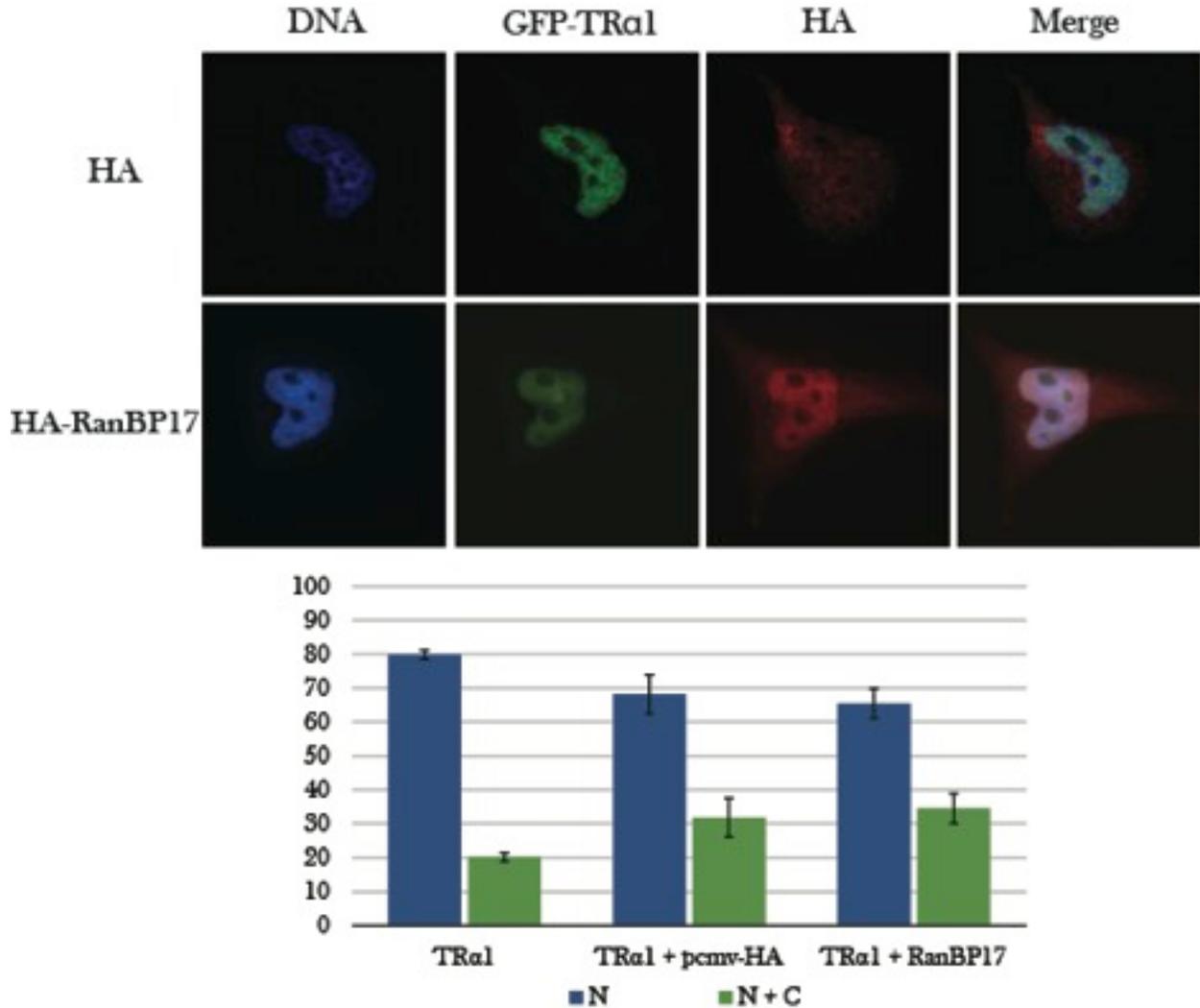


Figure 8: Overexpression of RanBP17 does not promote nuclear export of TRα1
 HeLa cells were transiently transfected with GFP-TRα1 expression plasmid and either HA or HA-RanBP17 expression plasmid. After approximately 24 hours the HeLa cells were fixed. The cells were then immunostained with anti-HA primary and Texas Red Anti-Rabbit IgG(H+L) secondary antibodies (red), before they were analyzed by fluorescence microscopy. GFP-TRα1 is visualized as green, while DAPI-stained nuclei are visualized as blue. The bar graph summarizes the distribution of GFP-TRα1 in HeLa cells overexpressing RanBP17, based on categories of primarily nuclear (N) or nuclear and cytoplasmic (N+C). Error bars are SEM and Chi-square had a p-value < 0.001 with three replicate experiments of three slides each with at least 100 cells scored per slide.

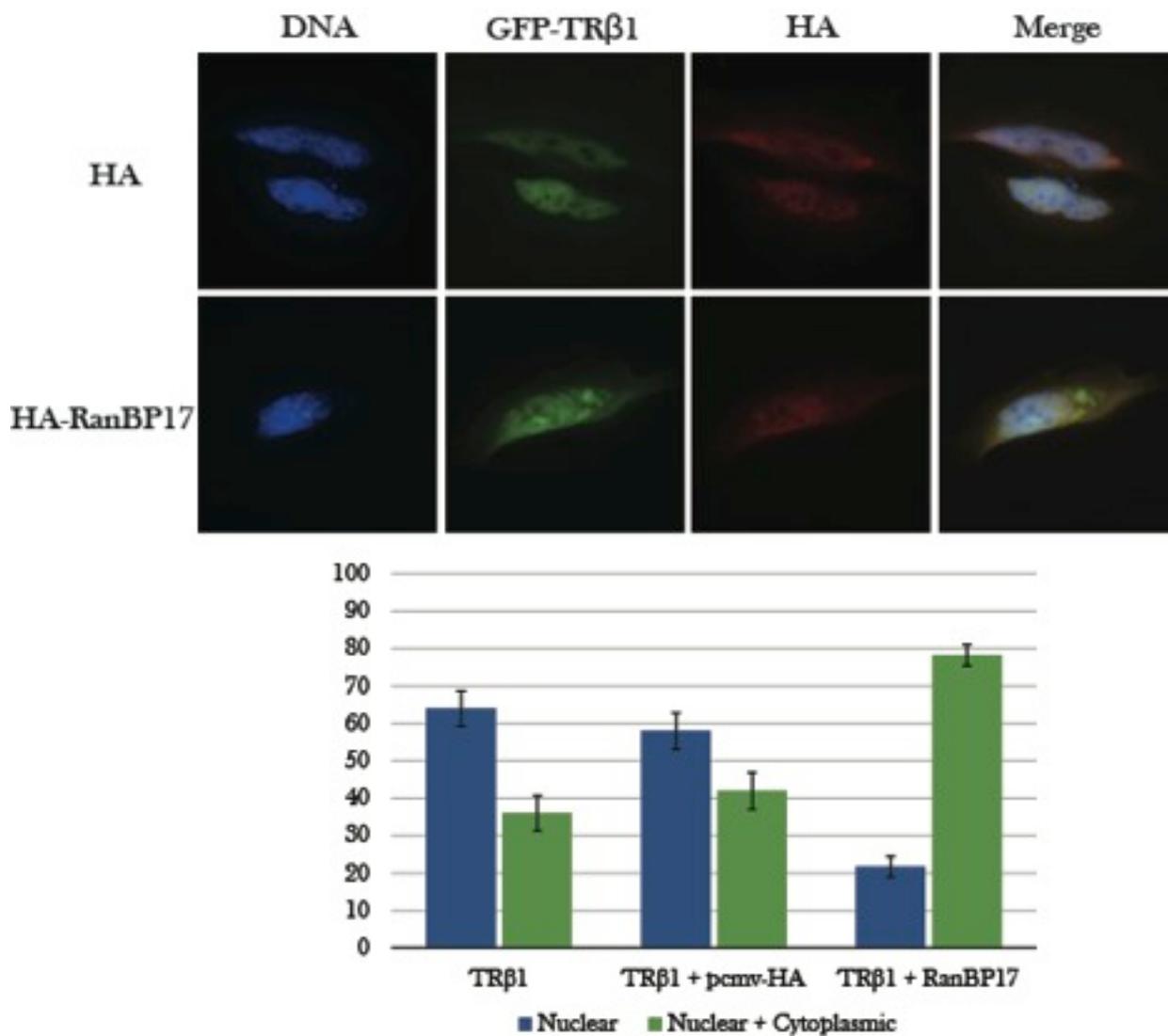


Figure 9: Overexpression of RanBP17 promotes nuclear export of TRβ1

Parallel experiments were conducted with GFP-TRβ1 expression plasmid and either HA or HA-RanBP17 expression plasmids. The bar graph summarizes the distribution of GFP-TRβ1 in HeLa cells overexpressing HA-RanBP17, based on categories of primarily nuclear (N) or nuclear and cytoplasmic (N+C). Error bars are SEM and Chi-square had a p-value < 0.001 with three replicate experiments of three slides each with at least 100 cells scored per slide.

TR β 1 and HA (control), there was a slight shift towards a more cytosolic distribution; 58.0% (\pm 1.6%) of cells had a nuclear distribution of TR β 1, and 42.0% (\pm 1.6%) of cells had a nuclear and cytosolic localization. When cells were expressing GFP-TR β 1 and HA-RanBP17, there was a dramatic shift towards a more cytosolic distribution; only 21.8% (\pm 4.9%) of cells had a nuclear distribution of TR β 1, while 78.3% (\pm 2.8%) of cells had a nuclear and cytosolic localization (Figure 9). Taken together, these results suggest that TR β 1 nuclear export can be mediated by RanBP17; however, this export pathway is not available to TR α 1.

OVEREXPRESSION OF RanBP17 REVEALS INCONCLUSIVE RESULTS CONCERNING TR-MEDIATED CAT REPORTER GENE EXPRESSION

To further our understanding of RanBP17 export, we thus used a CAT reporter gene under the positive control of a TRE to examine the transcriptional activity of GFP-TR (α 1 or β 1) (Figure 5). HeLa cells were transfected with expression plasmids for GFP-TR (α 1 or β 1), tk-TREpCAT reporter plasmid, and either HA-RanBP17, or HA as a control. After 12 hour incubation with 100nM of T₃, whole cell lysates were used for CAT ELISAs to determine CAT protein levels. When RanBP17 was overexpressed in the presence of T₃, very low levels of the CAT enzyme levels were detected. The amount of protein quantified for both TR α 1 or TR β 1 was very low, and therefore conclusions cannot be made about RanBP17 mediated export of TR.

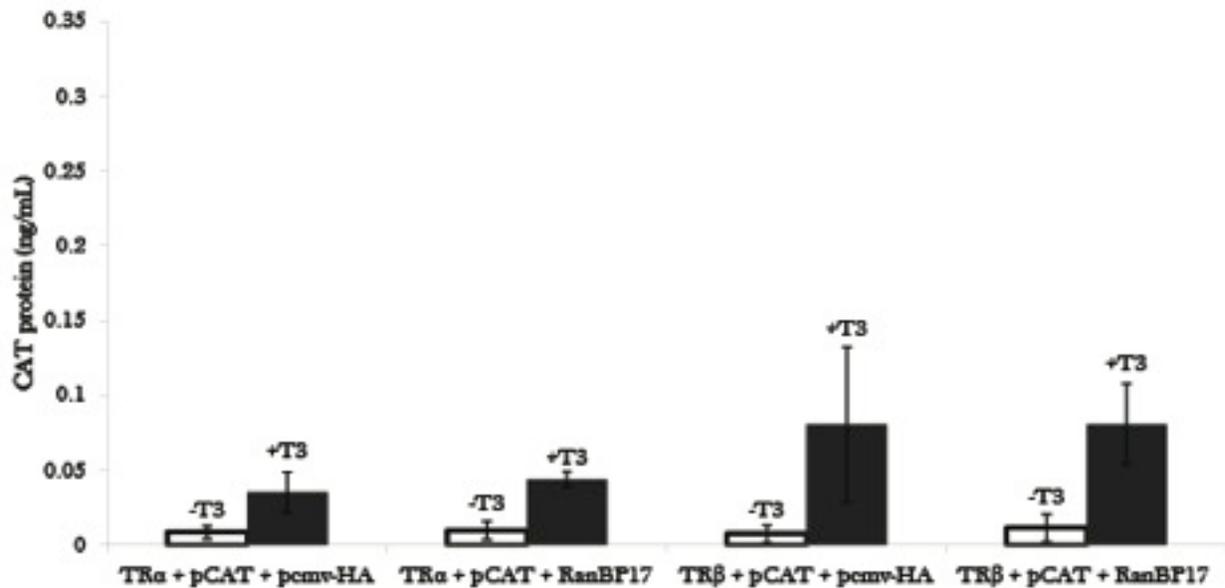


Figure 10: Low protein concentration reveals inconclusive results in overexpression of RanBP17, TR, and TRE CAT experiments.

HA or HA-RanBP17 expression plasmids, GFP-TR($\alpha 1$ or $\beta 1$) expression plasmids, and tk-TREp-CAT reporter plasmids were overexpressed through transient transfection in HeLa cells. CAT ELISAs were conducted to measure CAT protein levels in whole cell extracts 12 hours after addition of 100nM T_3 . Reporter gene expression (CAT protein) was measured in ng/mL ($n=4$). As a control, when GFP-TR($\alpha 1$ or $\beta 1$) expression plasmids were co-transfected with HA-RanBP17 in the absence of tk-TREp-CAT, there was no measured CAT protein (data not shown). The error bars in the bar chart represent SEMs. Overlapping error bars and overall low concentration of CAT protein conclude inconclusive results.

DISCUSSION

We have previously shown that TR can be exported from the nucleus through a CRM1-dependent or CRM1-independent pathway (Grespin et al., 2008; Mavinakere et al., 2012). We have also shown through RNA interference (RNAi) and fluorescence recovery after photobleaching (FRAP) that export of TR is inhibited when XPO5 and exportin 7 (XPO7) are knocked down, suggesting that XPO5 and XPO7 are potential export pathways used by TR (Subramanian, 2012). Here, we extended these findings to show that overexpression of XPO5 causes a shift in TR subcellular localization from the nucleus to the cytoplasm, which leads to a decrease in transcription of a TR-mediated CAT reporter gene. Using TR constructs with only one NES, we showed that XPO5 requires the helix 12 NES to mediate this shift in the subcellular localization of TR to the cytoplasm. In contrast, XPO5 had no effect on the subcellular distribution pattern of a construct containing the NES motif located in the helix 3-helix 6 region of TR. We also investigated the role of RanBP17 in TR nuclear export, as it shares homology with CRM1 and is most closely related to XPO7 (Koch et al., 2000). Interestingly, we showed that overexpression of RanBP17 causes a more cytosolic shift in the TR β 1 distribution, but has no impact on TR α 1 localization.

XPO5 MEDIATES TR NUCLEAR EXPORT

CRM1 has been described as the general export factor for NRs, but CRM1-independent pathways have also been characterized. TR has been shown previously to use a CRM1-calreticulin cooperative pathway (Grespin et al., 2008), which is consistent with other NRs that can use the calreticulin pathway such as the glucocorticoid receptor (Holaska et al., 2001). Overexpression experiments and CAT ELISA assays supported the hypothesis that XPO5 is important in mediating the nuclear export pathway of TR. Taken together, prior RNAi

experiments (Subramanian, 2012) and the results reported here provide evidence that XPO5 is important in the TR nuclear export pathway. Other known cargo for XPO5 includes tRNA, tRNA bound to eukaryotic elongation factor 1A, interleukin enhancer binding factor, microRNA, and double stranded RNA in a sequence independent manner (Calado et al., 2002; Brownawell and Macara, 2002; Yi et al., 2003; Bohnsack et al., 2004). These results classify TR as a new cargo of XPO5. XPO5 was first seen to mediate AR nuclear export through *in vitro* export pathway assays, where the particular NES involved was not described (Shank et al., 2008). A separate study by Saporita et al. (2003) describes a novel NES in ligand-binding-domain of AR, which is CRM1-independent and insensitive to leptomycin B; however, no studies were done to see if there was an interaction with this NES and XPO5. Furthermore, Saporita et al. (2003) explained that steroid receptors lack leucine rich sequences as seen in a classical CRM1 NES, suggesting an evolutionarily link for why TR would have a nuclear export pathway facilitated by XPO5, as XPO5 also mediates the export of the androgen receptor.

XPO5 TRANSPORTS TR USING THE NES LOCATED AT HELIX 12

The multiple NESs of TR have been described as CRM1-dependent and CRM1-independent. Mutagenesis studies by Mavinakere et al. (2012) discovered two novel CRM1-independent NESs located in TR either within helix 3, helix 6, or helix 12. Since there exists more than one possible CRM1-independent NES, it was hypothesized that XPO5 would transport TR using one NES over another. Through overexpression experiments, this hypothesis was supported. There was a significant shift of NES-H12-containing construct towards a greater cytosolic distribution. However, there was no significant shift in distribution of the NES-H3/H6-containing construct. These results suggest that XPO5 interacts either directly or indirectly with the NES in TR located in helix 12 of the ligand-binding domain. While less is known about

multiple export pathways used by one NR, the NLS-1 in the glucocorticoid receptor has been characterized to use three nuclear import pathways: importin α/β , importin 7, or importin 8 (Pemberton and Paschal, 2005; Kumar et al., 2006). Therefore, it is realistic to predict that other NRs with multiple NESs would use multiple nuclear export pathways.

A ROLE FOR RanBP17 IN TR β 1 NUCLEAR EXPORT

RanBP17 is currently poorly characterized, except that it is closely related to RanBP16/XPO7 of the importin- β superfamily (Koch et al., 2000) and shares sequence homology with CRM1 (Lee et al., 2010, Kutay, 2000). Previous research with RNAi knockdown has shown that RanBP16/exportin-7 may play a role in mediating TR nuclear export (Subramanian, 2012). Given the homology with other exportins used by TR, RanBP17 was examined as a potential mediator of TR nuclear export. Overexpression experiments with TR α 1/ β 1 and RanBP17 expression plasmids revealed that RanBP17 promotes TR β 1 nuclear export, as seen through a shift towards a greater cytosolic distribution of TR β 1 in the presence of RanBP17. However, RanBP17 had no effect on the subcellular distribution of TR α 1, and CAT ELISAs were inconclusive concerning RanBP17 as a possible export pathway for TR. These results may be inconclusive due to a faulty microplate reader, which has since been replaced or the concentration of CAT protein may be too little to be quantified. Nevertheless, the distinction between differences in gene regulation of the two isoforms (TR α 1/ β 1) as seen through the cell fixation experiments could be due to differential distribution of certain export pathways and isoforms (TR α 1/TR β 1) throughout the body. An example of differential isoform distribution of TR is seen in Forrest et al. (2006), where double knockout TR β mutant mice showed that only TR β is necessary for the critical function of the pituitary-thyroid axis.

SIGNIFICANCE

Nuclear export of TR has physiological effects on T₃ controlled gene expression. If TR is not physically located in the nucleus, then gene expression cannot be turned off or on. When TR is in the nucleus, it can change gene expression without a ligand present, because TR can act as a transcription activator when bound to T₃, but TR can also act as a transcription repressor when no ligand is bound (McKenna et al., 1999). Furthermore, nuclear export is an important tool to understand TR regulation, because certain isoforms have been shown to have different roles when localized in the cytoplasm. For example, TR β has been seen to directly change gene expression of transcription factor hypoxia-inducible factor alpha, while located in the cytoplasm (Moeller et al, 2006). Additionally, TR α located in the cytoplasm can up regulate the synthesis proteins in cardiomyocytes by interacting with phosphatidyl-inositol 3-kinase (Davis et al., 2008). Davis et al. (2008) also speculated further non-genomic effects of cytoplasmic TR in signal transduction pathways directed by thyroid hormone.

While post translational modifications are important in fine tuning gene expression in a cell, mislocalization can have wide reaching effects on disease, as seen in tissue areas of high nuclear receptor activity, such as breast, ovary, pancreas, intestine, prostate, and lung (O'Malley and Kumar, 2009). Specifically, when TR mislocalizes, resistance to thyroid hormone, changes in metabolism, and thyroid cancer can occur (Cheng et al., 2010). Mislocalization of TR has wide reaching effects, making the nuclear export pathways a key area of research. Further insight gained from the regulation of nuclear export of TR will ultimately enhance understanding of the role of TR in growth and development.

CONCLUSIONS

Our results show the ability of TR to use a CRM1-independent pathway, and thus our data begin to encompass the complexity of TR shuttling. In conclusion, results presented here suggest that XPO5 can mediate TR nuclear export via the NES located in helix 12 of the ligand binding domain. Further, RanBP17 may play a role in nuclear export of TR β 1; however, replicate CAT ELISA experiments are required to confirm this finding. Investigation of the role of other exportins, such as RanBP16/XPO7, is needed to fully characterize the multiple export pathways followed by TR.

FUTURE DIRECTIONS

CAT ELISA experiments with TR and RanBP17 need to be performed again; preliminary results were inconclusive. If RanBP17 causes a decrease in the amount of TR β 1-mediated CAT reporter gene expression, then these experiments will provide additional evidence for a role for RanBP17 in nuclear export. Additionally, the effect of RanBP17 on TR nuclear export has not been characterized through and fluorescence recovery after photobleaching (FRAP). The shuttling kinetics can be measured through knockdown experiments with short hairpin RNA, to inhibit RanBP17, and followed by FRAP. The ability for the photobleached nucleus to recover would denote to what extent the cell relies on RanBP17 for export of TR β 1.

The sister exportin to RanBP17 is RanBP16, also known as XPO7. Since these two exportins share homology, localization assays in the presence of XPO7 should be considered for future research. If these experiments yield interesting export results, CAT ELISAs should also be performed to confirm TR export and the related effect on transcription quantitatively.

Further XPO5 experiments should encompass protein-protein binding assays. Our results suggest that XPO5 interacts with the NES located at helix 12; however, we have yet to

show how XPO5 interacts with the NES, whether directly or indirectly. Pull-down assays and coimmunoprecipitation assays with XPO5, full-length TR, and NES constructs should be performed. If successful, these experiments will elucidate if there is an indirect or direct binding between XPO5 and TR.

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