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Multiple exportins influence thyroid hormone receptor localization

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ABSTRACT

The thyroid hormone receptor (TR) undergoes nucleocytoplasmic shuttling and regulates target genes involved in metabolism and development. Previously, we showed that TR follows a CRM1/calreticulin-mediated nuclear export pathway. However, two lines of evidence suggest TR also follows another pathway: export is only partially blocked by leptomycin B (LMB), a CRM1-specific inhibitor; and we identified nuclear export signals in TR that are LMB-resistant. To determine whether other exportins are involved in TR shuttling, we used RNA interference and fluorescence recovery after photobleaching assays in transfected cells. Knockdown of exportins 4, 5, and 7 altered TR shuttling dynamics, and when exportins 5 and 7 were overexpressed, TR distribution shifted toward the cytosol. To further assess the effects of exportin overexpression, we examined transactivation of a TR-responsive reporter gene. Our data indicate that multiple exportins influence TR localization, highlighting a fine balance of nuclear import, retention, and export that modulates TR function.

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1. Introduction

Thyroid hormone (triiodothyronine, T3) is important in regulating genes responsible for metabolism, growth, and development. Encoded by two different genes, thyroid hormone receptors TRα1 and TRβ1 respond to T3 levels by activating or repressing target gene expression. Although primarily found in the nucleus at steady-state, TRα1 and TRβ1 can rapidly shuttle between the nucleus and cytoplasm (Baumann et al., 2001; Bunn et al., 2001; Grespin et al., 2008). Nuclear import and export of proteins occurs through the nuclear pore complexes, mediated by members of the karyopherin β family called importins and exportins, respectively (Kimura and Imamoto, 2014; Pemberton and Paschal, 2005). By coupling mutagenesis and localization studies, nuclear localization signal (NLS) and nuclear export signal (NES) motifs that interact with the transport machinery have been found in conserved domains of members of the nuclear receptor superfamily (Black et al., 2004; Holaska et al., 2002; Kanno et al., 2005, 2007; Liu and DeFranco, 2000; Lombardi et al., 2008; Mavinakere et al., 2012; Nguyen et al., 2009; Pemberton and Paschal, 2005; Picard and Yamamoto, 1987; Saporita et al., 2003; Sorokin et al., 2007; Umemoto and Fujiki, 2012). This transport process provides a central regulatory point for coordinating cell signaling and gene expression.

From our prior studies, an increasingly complex picture has emerged of the intricate molecular mechanisms that regulate trafficking and function of TRα1 and TRβ1 (collectively referred to as TR hereinafter for simplicity). Previously, we showed that TR can exit the nucleus by a pathway mediated by the export factor CRM1 (chromosome region maintenance 1), also known as exportin 1, in cooperation with calreticulin (Grespin et al., 2008); however, the exact interaction and mechanism remain unclear. Two main lines of evidence suggested that TR might also follow a CRM1/calreticulin-independent nuclear export pathway. First, under conditions in which TR still shuttles, shuttling of p53 and the oncoprotein v-ErbA is completely blocked in the presence of the CRM1-specific inhibitor leptomycin B (LMB) (Bunn et al., 2001; DeLong et al., 2004). Also, during fluorescence recovery after photobleaching (FRAP) experiments, when one nucleus in a multinucleate HeLa cell was photobleached, recovery of fluorescence in the bleached nucleus in the presence of LMB was reduced by only 60%, relative to recovery in the absence of LMB (Grespin et al., 2008). Second, no CRM1-dependent NES in TR has yet been characterized (Mavinakere et al., 2012). Indeed, our studies showed that TR interacts directly with calreticulin but complex formation with CRM1 was not detectable in pull-down assays (Grespin et al., 2008). In an effort to identify and clarify the mode of TR nuclear export, we previously carried...
out a comprehensive analysis of TR to screen for NES motifs. We
identified a region spanning helices 3 and 6 of the ligand-binding
domain, that either houses two monopartite NESs or a single, bi-
partite NES, and we fully characterized another NES in helix 12 of
the ligand-binding domain. These NES motifs were able to export
a nucleus-localized fusion protein to the cytosol (Mavinakere et al.,
2012). Intriguingly, these NES motifs were shown to be insensi-
tive to LMB. In the presence of LMB, they were still able to direct
the fusion protein to the cytosol, suggesting that they follow a CRM1-
independent export pathway (Mavinakere et al., 2012).

In the present study, we sought to determine whether other exportins are involved in this alternative nuclear export pathway of
TR, and to determine their relative contributions to TR export
overall. To this end, we coupled RNA interference (RNAi) with FRAP
experiments in live HeLa cells. Shuttling dynamics of TR were as-
sumed upon knockdown of transportins 1 and 2, and exportins, 4,
5, 6, and 7. Additionally, we used overexpression assays and T3-
responsive reporter gene assays to further assess the role of a panel
from each set of four (see Section 2.4).

2. Methods

2.1. Plasmids

The plasmid pGFP-TRα1 encodes a functional green fluores-
cent protein (GFP)-tagged rat TRα1 fusion protein (Bunn et al., 2001).
pGFP-TRβ1 encodes a functional GFP-tagged human TRβ1 (Mavinakere et al., 2012). Pre-designed SureSilencing™ short hairpin
RNA (shRNA) plasmid sets consisting of four different shRNA expression plasmids for each target mRNA were purchased from
SABioscience (Frederick, MD) for human transportin 1 (TNP01), transportin 2 (TNP02), exportin 4 (XPO4), exportin 5 (XPO5), exportin
6 (XPO6), exportin 7 (XPO7), and a scrambled sequence negative
control, pk-Myc-exportin 5, pcMV-Myc, and pcMV-HA were ob-
tained from Addgene (Cambridge, MA), Clontech Laboratories, Inc.
(Mountain View, CA), and BD Biosciences (San Jose, CA), respec-
tively. The HA-tagged exportin 7 expression plasmid (pMT2SM-
RanBP16) was a gift from C. Smas (University of Toledo College
of Medicine, Ohio). The mCherry-tagged exportin 4 expression plasmid
(pmCherry-XPO4) was obtained from GenScript (Piscataway, NJ) and
pmCherry-C1 was from Clontech. 2xDR4-SV40-Luc was a gift from
J. L. Jameson (Northwestern University) and consists of two copies
of a positive, direct repeat TRE (DR+4) in the firefly luciferase vector
pGL3. pGL4.74 encodes Renilla luciferase (Promega, Madison, WI).

2.2. Fluorescence recovery after photobleaching (FRAP)

HeLa cells (ATCC, #CCL-2) were cultured in minimum essential
medium (MEM) supplemented with 10% fetal bovine serum (Life
Technologies, Grand Island, NY) at 37 °C under 5% CO2 and 98% hu-
midity. Cells were seeded at 2.0–2.5 × 106 cells per 3-cm dish with a
cover glass bottom (MatTek Corporation, Ashland, MA). Twenty
four hours after seeding, cells were co-transfected with 1 μg GFP-
TRα1 expression plasmid, and 1 μg of the appropriate target-
specific or control shRNA expression plasmids, using the two shRNAs
from each set of four (see Section 2.1) that showed the greatest
knockdown efficacy as assessed by quantitative PCR (see Section 2.4).
Transfection medium containing Lipofectamine 2000 (Life Tech-
nologies) was replaced with complete medium 9 h post-transfection.
Twenty seven hours post-transfection, cells were prepared for live-
cell imaging: cells were incubated in 2 mL of complete media
containing 100 μg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO),
100 units/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL
wheat germ agglutinin conjugated to Alexa Fluor® 350 (Life Tech-
nologies). Cells were washed twice with Dulbecco’s phosphate-
buffered saline and imaged. During the experiment, cells were
incubated in MEM-α without phenol red, containing 50 μg/mL cy-
cloheximide, 50 units/mL penicillin, and 50 μg/mL streptomycin.

In preliminary studies, we tested a range of post-transfection incuba-
tion times (17 h, 24 h, 27 h, and 30 h), varied the amount of
Lipofectamine 2000 and the time cells were exposed to the reagent,
selected for knockdown cells with puromycin, and varied the shRNA
plasmid amounts and combinations. The conditions described earlier
were determined to have high transfection efficiency (70–80% of
cells were transfected), effectively reduce the levels of exportins in
cells (at least 50% knockdown), while still retaining cell viability.
Altered conditions either decreased transfection efficiency, de-
creased knockdown efficiency, or led to increased cell mortality. Cell
mortality was assessed by visual inspection of the number of ad-
herent cells prior to transfection, compared with the number of cells
remaining adhered post-transfection, with the standard set at >60%
retention.

All FRAP experiments were performed in an OkoLab Incuba-
tion System (Warner Instruments, Inc., Hamden, CT) at 37 °C under
5% CO2. Images were collected from an inverted Nikon A1RSi con-
foveal microscope Ti-E-PFS using a 40× water objective (Nikon Inc.,
Melville, NY). The 488-nm line of a krypton–argon laser with a band-
pass 525/50 nm emission filter was used for GFP detection; the 405-
nm line with a band-pass 450/50 nm emission filter was used for Alexa
Fluor® 350 detection. Images were obtained using the stimulation/
bleaching acquisition module of NIS-Elements AR (Nikon). 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 8–20% laser power from the 405 nm line. One nucleus within
the multinucleated cell was exposed at 100% laser power for 10–12 s
using the 488 nm line. Post-bleach sequential images were then taken
every 5 min for 24 cycles at the lower laser intensities noted earlier.
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 back-
ground brightness levels during each experiment. Intensity values
were subsequently normalized so that the total fluorescence within
each multinucleated cell after bleaching was equal to 1.0 (arbi-
trary units). After normalization, convergence of the representative
curves for bleached and unbleached nuclei toward one another rep-
resents the degree of fluorescence equilibration between these
compartments. When one bleached and one unbleached nucleus
are present, complete equilibration occurs at 0.5 fluorescence units
(Grespin et al., 2008).
was used at 1:50. Coverslips were mounted in Fluoro-Gel II containing the DNA counter stain 4’, 6-diamidino-2’-phenylindole (DAPI, 0.5 μg/mL) (Electron Microscopy Sciences, Hatfield, PA).

Images were analyzed with an inverted Nikon Eclipse TE 2000-E fluorescence microscope. A Nikon Ultraviolet Excitation: UV-2E/C filter block for DAPI visualization, a Blue Excitation: B-2E/C filter block for GFP, and a Red Excitation: T-2E/C filter block for the Myc or HA tag were used with a Nikon Plan Apo 40x objective. NIS-Elements AR software was used for image acquisition and primary image processing. Cells were scored blind, without knowledge of the treatment conditions. The slides’ original labels were removed and replaced with random number labels by another lab member, who made a key and kept it secure until the scoring was completed and data were analyzed. All experiments consisted of a minimum of 3 replicates and at least 100–300 cells were scored per replicate. The state of the nuclei was assessed by visually examining the integrity and morphology of each DAPI-stained nucleus; only cells with intact nuclei were scored. Intracellular distribution patterns of TR in cells transfected with pGFP-TRα1 or pGFP-TRβ1 and pCMV-Myc or pMyc-Exp5 were scored into two categories: primarily nuclear (N), or distributions ranging from nuclear accumulation but a clearly visible cytosolic population to a whole cell distribution (N + C/WC). Intra-cellular distribution patterns of TR in cells transfected with pGFP-TRα1 or pGFP-TRβ1 and pmCherry-XPO4, pmCherry, pCMV-HA, or HA-exportin 7 (RanBP16) plasmids were scored into three categories: primarily nuclear (N), nuclear accumulation and a clearly visible cytosolic population (N + C); or whole cell, where the nucleus was not distinct (WC) (see Supplementary Fig. S1). Data were quantified as the percentage of cells in a given category (e.g., % of cells with a primarily nuclear distribution of TR).

2.4. Validation of RNA interference (RNAi) by quantitative PCR (qPCR)

HeLa cells seeded at 6–7 × 10^5 cells per 100-mm vented plate were transfected with 10 μg target-specific or control shRNA expression plasmids, using Lipofectamine 2000. Medium was replaced with complete medium 9 h post-transfection. Twenty seven hours post-transfection, RNA was extracted using the Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s specifications, with the exception that the DNase I digestion was extended to 30 min. RNA quality was analyzed using an RNA 6000 Pico Total RNA Assay and Agilent 2100 BioAnalyzer’s Lab-on-a-Chip Technology (Santa Clara, CA). CDNA was synthesized using the SABioscience RT² First Strand Kit, following the manufacturer’s specifications. Quantitative PCR (qPCR) was performed using the Real-Time RT² qPCR Primer Assay (SABiosciences) with RT² Real-Time™ SYBR Green/Fluorescein qPCR master mix and SABioscience validated primers specific for each exportin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. qPCR data were analyzed by the ΔΔCt (Livak) method (Livak and Schmittgen, 2001) using the Applied Biosystems StepOne Software Version 2.1 (Life Technologies).

2.5. Western blotting

Twenty seven hours post-transfection, HeLa cells were harvested in lysis buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 10 mM NaF, 10% glycerol, 1% Nonidet P-40 (Calbiochem, San Diego, CA), Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (1 tablet per 10 mL; Roche Diagnostics, Indianapolis, IN)), Lysates were centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatant protein concentration was determined using a NanoDrop® ND-1000 Full-spectrum UV/Vis Spectrophotometer. Lysates (40–60 μg of protein per lane) were separated by 8% SDS–PAGE and transferred to PVDF membrane using the iBlot Dry Blotting System (Life Technologies).

The membranes were incubated overnight at 4 °C in blocking solution (Tris-buffered saline containing 0.1% Tween 20 [T-TBS], 1% bovine serum albumin). After 4–6 washes with T-TBS at room temperature, the membranes were incubated with primary antibodies for 1.5–2 h. All antibodies were incubated separately and used at the following concentrations: anti-β-tubulin (Santa Cruz Biotechnology Inc., Dallas, TX), 1:200; anti-exportin 5 (Abcam), 1:400; anti-transportin 1 (Abcam), 2.0 μg/mL; anti-transportin 2 (Santa Cruz), 1:1000; anti-exportin 4 (Santa Cruz), 1:500; anti-exportin 5 (Santa Cruz), 1:1000; anti-exportin 7 (Abcam), 0.5 μg/mL; and anti-GAPDH (Santa Cruz), 1:5000. Blots were then washed 4–6 times with T-TBS and incubated with the appropriate secondary antibody for 1.25 hours in blocking solution. Secondary antibodies were used at the following concentrations: horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare Life Sciences, Pittsburgh, PA), 1:25,000; HRP-sheep anti-mouse IgG (GE Healthcare Life Sciences), 1:25,000; and HRP-mouse anti-goat IgG (Santa Cruz), 1:10,000 or 1:25,000. Subsequently, blots were washed 6–8 times in T-TBS, followed by chemiluminescent detection using ECL Prime detection reagent (GE Healthcare Life Sciences). Protein size was monitored using Pre-Stained Kaleidoscope Protein Standards (Bio-Rad). X-ray films were quantified by scanning densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

2.6. Luciferase reporter gene assay

HeLa cells were seeded at 2.0 × 10^4 per well in a 96-well plate (PerkinElmer, Waltham, MA). Seventeen hours after seeding, cells were transiently transfected with 100 ng DNA, containing 25 ng each of expression plasmids for GFP-TRα1 or GFP-TRβ1, TRE (DR4)-firefly luciferase reporter, Renilla luciferase internal control, and mCherry, mCherry-XPO4, Myc, Myc-XPO5, HA, or HA-XPO7. Transfection medium was replaced with complete medium 6 h post-transfection. Twelve hours post-transfection, complete medium was replaced with 100 μL MEM containing 10% charcoal-dextran stripped FBS (Life Technologies), supplemented or not with 100 nM T3. After an additional 12 h, a Dual-Glo® Luciferase Assay (Promega) was performed, according to the manufacturer’s protocol, using 100 μL of reagent per well.

2.7. Statistical analyses

Data represent the mean ± 1 SEM of at least three independent experiments. Statistical differences between two groups were determined using an unpaired Student’s t test with the two-tailed P value. Results were considered significant at P < 0.05.

3. Results

3.1. Knockdown of exportins 4, 5, and 7 slows nucleocytoplasmic shuttling of TRα1

Prior studies pointed to the possibility that, in addition to following a cooperative CRM1/calreticulin-dependent export pathway during nucleocytoplasmic shuttling, TR may also follow an alternative nuclear export pathway that does not rely on CRM1 (Bunn et al., 2001; Grespin et al., 2008; Mavinakere et al., 2012). To determine whether other exportins play a role in mediating TR nuclear export, we coupled in vivo approaches using FRAP and RNAi in transfected HeLa cells to examine how knockdown of individual exportins impacts the shuttling dynamics of TRα1. Since TRα1 is primarily nuclear at steady-state, but shuttles between the nucleus and the cytosol, knockdown of an essential export factor would be predicted to result in decreased nuclear export of TRα1. This effect would be visualized as greater retention of fluorescence in the unbleached nucleus, along with a significantly slower recovery of fluorescence...
to the bleached nucleus during a FRAP assay. It is important to note, however, that it was not expected that cells would ever show complete inhibition of TRα1 shuttling since RNAi leaves a portion of the target mRNA and protein in cells, and there are likely multiple pathways for export. In addition, if knockdown of a bidirectional transport factor affected nuclear import instead, this defect in import would be visualized as an accumulation of TRα1 in the cytosol.

To begin, shRNA-induced knockdown of target exportin mRNA and protein levels were validated by qPCR and western blotting, respectively. The levels of exportin mRNA in the presence of target shRNA were reduced by ≥75%, relative to the scrambled shRNA control (control mRNA expression was set at 100%) (Fig. 1A). Exportin protein levels were reduced relative to the scrambled control, on average, as follows: transportin 1, 31%; transportin 2, 56%; exportin 4, 41%; exportin 5, 30%; and exportin 7, 40% (Fig. 1B). We were unable to acquire a viable antibody for exportin 6, so in this case validation was restricted to qPCR. Taken together, these levels of knockdown confirm the efficacy of the RNAi system.

Next, we compared shuttling of GFP-TRα1 with GFP-TRβ1 under standard conditions, in the absence of shRNA expression (Fig. 2A, Supplementary Videos S1 and S2). To confirm that experiments were conducted in a single cell with multiple nuclei and not nuclei in adjacent separate cells, transfected HeLa cells were incubated before visualization with fluorescent-tagged wheat germ agglutinin (WGA), an external plasma membrane marker for live-cell imaging (Fig. 2A). All FRAP experiments were also performed in the presence of cycloheximide to ensure that the fluorescence recovery of GFP in bleached nuclei was not due to de novo protein synthesis. As shown in Fig. 2A, both TRα1 and TRβ1 undergo shuttling; however, for TRβ1 a faint cytosolic population is visible at t = 0, while TRα1 appears entirely nuclear. Thus, to avoid any ambiguity in interpreting FRAP results, we only performed knockdown assays with TRα1. To determine the effect of exportin knockdown on the dynamic shuttling of TRα1, HeLa cells were cotransfected with GFP-TRα1 and exportin-specific shRNA expression plasmids.

We first tested whether transportins 1 and 2 play a role in TRα1 nuclear export (Fig. 2B). Originally proposed to mediate bidirectional transport, more recent studies maintain that transportins 1 and 2 are restricted to mediating nuclear import of RNA-binding proteins that function as splicing regulators (Twyffels et al., 2014). Therefore, we predicted that the transportins would not be involved in TRα1 shuttling. For quantitation of FRAP (Fig. 3), bleached and unbleached nuclei were each considered as independent regions of interest. Intensity values were normalized so that the total fluorescence within each multinucleated cell after bleaching was equal to 1.0 (arbitrary units). After normalization, convergence of the representative curves for bleached and unbleached nuclei toward one another represents the degree of fluorescence equilibration between these compartments. When one bleached and one unbleached nucleus are present, complete equilibration occurs at 0.5 fluorescence units (Grespin et al., 2008). Recovery of fluorescence to bleached nuclei within live cells transfected with a scrambled control shRNA was measured at, on average, 51% fluorescence equilibration at 60 min and 72% at 120 min (Fig. 3). As expected, comparable shuttling dynamics were observed for cells transfected with transportin 1 shRNA, relative to the control (60 min, P = 0.89; 120 min, P = 0.59). At 60 min and 120 min, transportin 1 knockdown cells showed, on average, 49% and 65% fluorescence equilibration with unbleached nuclei, respectively. Similarly, there was no significant difference in TRα1 shuttling between the control and

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**Fig. 1.** Validation of shRNA knockdown of target gene mRNA and protein levels. (A) HeLa cells were transiently transfected with a panel of exportin-specific shRNA expression plasmids, as indicated, or a scrambled shRNA plasmid as a control. qPCR was used to confirm knockdown of exportin mRNA levels. Bars indicate the relative expression level of exportin mRNA in cells treated with exportin-specific shRNA versus control cells (control mRNA expression set at 100%), normalized to the levels of the housekeeping mRNA, GAPDH. Error bars indicate ±1 SEM (n = 3). TNPO1, transportin 1; TNPO2, transportin 2; XPO4, exportin 4; XPO5, exportin 5; XPO6, exportin 6; XPO7, exportin 7. (B) Western blot analysis was used to confirm knockdown of exportin protein levels relative to the control (control protein expression set at 100%), as indicated. Error bars indicate ±1 SEM (n = 3, TNPO1, XPO4, XPO5, XPO7; n = 5, TNPO2).
transportin 2 shRNA-transfected cells (60 min, $P = 0.68$; 120 min, $P = 0.78$). At 60 min and 120 min, transportin 2-knockdown cells showed, on average, 58% and 75% fluorescence equilibration with unbleached nuclei, respectively (Fig. 3). These data show that transportins 1 and 2 are not involved in nuclear retention or export of TRα1. Furthermore, no cytosolic accumulation of TRα1 was observed during the FRAP assay (Fig. 2B), indicating that knockdown of transportins 1 and 2 did not interfere with nuclear import of TRα1.

Exportin 4 is a bidirectional nuclear transport factor involved in nuclear export of translation initiation factor eIF-5A and transcriptional regulator Smad3 (Chook and Suel, 2011). Thus, we predicted this selective transporter would not play a role in promoting TRα1 nuclear export. At 60 min, there was no significant difference between TRα1 shuttling in control shRNA and exportin 4 shRNA-transfected cells (Figs. 2B and 3; Supplementary Videos S3 and S4) ($P = 0.06$), with recovery in bleached nuclei in knockdown cells measured at 34% fluorescence equilibration, on average. However, unexpectedly, at 120 min there was a significant difference in the amount of recovery relative to the control ($P = 0.04$); exportin 4 knockdown-cells only reached 52% fluorescence equilibration, on average (Fig. 3), suggesting that exportin 4 plays a role in TR nuclear export, either directly by facilitating exit of TRα1 from the nucleus or, indirectly by decreasing nuclear retention and thereby promoting interaction with other exportins. No cytosolic accumulation was observed (Fig. 2B), which if present would have been indicative of a defect in import, suggesting that exportin 4 does not play a role in mediating nuclear entry of TRα1.

The primary cargo of exportin 5 is microRNA (miRNA) precursors (Bohnssack et al., 2004; Lund et al., 2004); however, it also has been shown to mediate nuclear export of the androgen receptor,
another member of the nuclear receptor superfamily (Shank et al., 2008). Thus, we predicted that knockdown of exportin 5 would alter TRα1 shuttling dynamics. Instead, although there appeared to be a modest decrease in TRα1 shuttling between cells transfected with control shRNA and exportin 5 shRNA knockdown, this effect was not significant at either 60 min ($P = 0.12$) or 120 min ($P = 0.22$) (Figs. 2B and 3). Fluorescence equilibration was measured, on average, at 38% and 60% for 60 min and 120 min, respectively (Fig. 3). Since exportin 5 mediates export of pre-miRNA and shRNA from the nucleus, there is a trade-off between down-regulating exportin 5, but still getting enough pre-shRNA out of the nucleus for sustained knockdown of exportin 5 mRNA levels. 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 overexpressing exportin 5 in the presence of Myc-tagged exportin 5 shRNA could, albeit counterintuitively, enhance knockdown. First, we validated the utility of this approach. In the presence of XPO5 shRNA, exportin 5 protein levels were decreased compared to the control shRNA, where no knockdown occurred (Fig. 2C; see also Fig. 1B). In contrast, when exportin 5 is overexpressed, there was even greater knockdown of exportin 5 protein levels with target shRNA. After determining that more efficient knockdown occurs

![Fig. 3. Nucleocytoplasmic shuttling of TRα1 is slowed by knockdown of exportins 4, 5, and exportin 7. Fluorescence recovery graphs summarize the data from the replicate FRAP experiments described in Fig. 2B. Blue squares are the intensity within bleached nuclei and red diamonds are the intensity within unbleached nuclei. Intensity values were normalized so that the total fluorescence within each multinucleated cell after bleaching was equal to 1.0 (arbitrary units). Convergence of the curves for bleached and unbleached nuclei toward one another represents the degree of fluorescence equilibration between the nuclei. When one bleached and one unbleached nucleus are present, complete equilibration occurs at 0.5 fluorescence units. Error bars indicate ±1 SEM. *$P < 0.05$.](image-url)
when overexpressing exportin 5, we ran parallel FRAP experiments. In reduction in shuttling of TRα1 (Figs. 2B and 3, Supplementary Video S5) at 60 min (P = 0.03); however, after 120 min recovery was not significantly different from the control (P = 0.05). The fluorescence equilibrations from unbleached nuclei to bleached nuclei were, on average, 26% at 60 min and 49% at 120 min (Fig. 3).

Next, we predicted that knockdown of exportins 6 and 7 would not alter TRα1 shuttling dynamics for the following reasons. Exportin 6 is a specific transporter of nuclear actin (Dopie et al., 2012; Stuven et al., 2003) and, although exportin 7 binds diverse cargo, it had not been shown to be involved in export of other members of the nuclear receptor superfamily (Mingot et al., 2004). As predicted, knockdown of exportin 6 resulted in similar TRα1 shuttling dynamics compared to the scrambled control, at both 60 min (P = 0.44) and 120 min (P = 0.46) (Figs. 2B and 3). Fluorescent equilibration of bleached nuclei was measured, on average, at 43% and 63% for 60 min and 120 min, respectively. In contrast, knockdown of exportin 7 resulted in altered shuttling of TRα1 (Figs. 2B and 3, Supplementary Video S6). Fluorescent equilibrations were measured, on average, at 32% and 48%, for 60 min and 120 min, respectively. Differences were not significant at 60 min (P = 0.06); however, at 120 min there was a significant decrease in recovery (P = 0.03), compared with the control, suggesting that exportin 7 plays a role in TRα1 cellular localization, either directly via mediating nuclear export, or indirectly via increasing intranuclear mobility and access to the export machinery.

Finally, we predicted that dual knockdown with combinations of shRNA against exportins 4, 5, and 7 would have a greater impact than single knockdowns. However, when we tested dual knockdown of exportins 4 and 5, exportins 5 and 6 (as a control), and exportins 5 and 7, these combinations did not result in further shifts in the shuttling pattern of TRα1 (data not shown), although this could well be due to increased cell mortality. These exportins are required for trafficking of proteins involved in many vital cell processes and, since cellular miRNAs potentially regulate the expression of hundreds of genes, saturation of the RNAi pathway with exogenous shRNA also contribute to loss of cell viability (Castanotto and Rossi, 2009; Scherr and Eder, 2007). In addition, it is likely that the primarily nuclear location of TRα1 at steady-state limits how much the shuttling pattern can be altered over the time course of an experiment.

Taken together, these data provide evidence that in addition to CRM1 and calreticulin, exportins 4, 5, and 7 either directly or indirectly play a role in promoting nuclear export of TRα1. In contrast, transportins 1 and 2, and exportin 6 do not play a role, or at least not an essential role, in modulating TRα1 shuttling.

3.2. Overexpression of exportin 5 and exportin 7 promotes nuclear export of TRα1 and TRβ1

Having shown that nucleocytoplasmic shuttling of TRα1 is partially inhibited by knockdown of exportins 4, 5, or 7, we sought to ascertain whether their overexpression would alter the cellular localization of both TRα1 and TRβ1. We predicted that overexpression of exportins 4, 5, and 7 would cause a shift in the distribution of TRα1 and TRβ1, from the nucleus to the cytosol. To this end, HeLa cells were cotransfected with expression plasmids for GFP-tagged TRα1 or β1, and mCherry-tagged exportin 4, Myc-tagged exportin 5, or HA-tagged exportin 7, or mCherry, Myc tag, or HA tag, as controls. Distribution patterns were visualized by direct fluorescence or immunofluorescence microscopy (Figs. 4 and 5).

Consistent with our predictions, when exportin 5 was overexpressed, a significantly greater percentage of cells showed a shift toward a more cytosolic distribution for both TRα1 (P = 0.00001) and TRβ1 (P = 0.00006) (Fig. 4). In cells co-expressing GFP-TRα1 and the Myc tag (control), on average 90% of cells showed a primarily nuclear distribution of TRα1 (Fig. 4A). In contrast, in cells co-expressing GFP-TRα1 and Myc-tagged exportin 5, on average only 52% of cells showed a primarily nuclear distribution of TRα1 (Fig. 4A). In the cells expressing GFP-TRβ1 and the Myc tag, on average 76% of cells had a primarily nuclear distribution of TRβ1, compared with 34% of cells showing a primarily nuclear distribution of TRβ1 in the presence of Myc-tagged exportin 5 (Fig. 4B).

Interestingly, although knockdown of exportin 4 significantly slowed TR shuttling, overexpression of exportin 4 had no significant effect on the distribution patterns of either TRα1 (P = 1.00) or TRβ1 (P = 0.84) (Fig. 5A). In cells co-expressing GFP-TRα1 and mCherry (control) or mCherry-exportin 4, on average 77% of cells showed a nuclear localization of TRα1. In the cells co-expressing GFP-TRβ1 and mCherry or mCherry-exportin 4, on average 55% of cells showed a nuclear localization of TRβ1. Comparable results to exportin 5 were obtained when exportin 7 was overexpressed; a significantly greater percentage of cells showed a shift toward a more cytosolic distribution for both TRα1 and TRβ1, relative to the control (P = 0.00001) (Fig. 5B). In cells co-expressing GFP-TRα1 and the HA tag (control), on average 68% of cells showed a nuclear distribution of TRα1. In contrast, in cells co-expressing GFP-TRα1 and HA-tagged exportin 7, on average only 25% of cells showed a primarily nuclear distribution of TRα1. In the cells expressing GFP-TRβ1 and the HA tag, on average 59% of cells had a primarily nuclear distribution of TRβ1, compared with 22% of cells showing a primarily nuclear distribution of TRβ1 in the presence of HA-exportin 7.

3.3. Effect of enhanced nuclear export on TR-mediated ligand-independent and ligand-dependent gene expression

In the absence of Tα1, unliganded TRα1 and TRβ1 repress the expression of target genes that are under control of positive thyroid hormone response elements (TREs). In the presence of Tα1, the liganded receptors stimulate expression of these same genes. Thus, we sought to ascertain whether the cytosolic shift in the distribution of TRα1 and TRβ1 resulting from overexpression of exportins 5 and 7 would alter TR-mediated gene expression to a comparable extent. A firefly luciferase reporter gene under the control of a positive TRE (DR4) was used to examine ligand-dependent transactivation by TRα1 (Fig. 6A) and TRβ1 (Fig. 6B), in the presence of exportin 4 (no cytosolic shift), exportin 5, or exportin 7. Exportin overexpression was confirmed by western blot analysis (data not shown). In the absence of Tα1, for cells overexpressing mCherry-exportin 4 or Myc-exportin 5, there was no significant difference in relative luciferase activity by unliganded TRα1 or TRβ1, compared with luciferase activity in cells overexpressing mCherry and Myc controls (TRα1 +XPO4, P = 0.29; TRα1 +XPO5, P = 0.07; TRβ1 +XPO4, P = 0.36; TRβ1 +XPO5, P = 0.54). Similarly, there was no significant difference in repression of luciferase activity by unliganded TRβ1 in cells overexpressing HA-exportin 7, compared to HA alone (P = 0.31). In contrast, cells overexpressing HA-exportin 7 showed significantly greater luciferase reporter activity in the presence of unliganded TRα1, compared with HA alone (P = 0.04). On average, luciferase activity levels were increased 3.0-fold in the presence of exogenous exportin 7, relative to levels in the absence of exogenous exportin 7, suggesting that exportin 7 has wider effects than on TRα1 export alone.

We also examined the ability of liganded TR to stimulate TREF-luciferase reporter gene expression. In the presence of Tα1, fold stimulation of luciferase activity by liganded TRα1 and TRβ1 in cells overexpressing Myc-exportin 5 or HA-exportin 7 was not significantly different compared with fold stimulation in the presence of the Myc and HA tag controls (TRα1 +XPO5, P = 0.94; TRα1 +XPO7, P = 0.60; TRβ1 +XPO5, P = 0.41; TRβ1 +XPO7, P = 0.53), indicating that under these conditions a reduction in the percentage of cells with
primarily nuclear TRα1 or TRβ1 of 38–42% does not have a measurable impact on reporter gene stimulation. Likewise, fold stimulation by liganded TRβ1 in the presence of overexpressed mCherry-exportin 4 also was not significantly different from the control (P = 0.90). Interestingly, however, when TRα1 and mCherry-exportin 4 were co-expressed in the presence of T3, fold stimulation of luciferase was significantly increased, relative to mCherry alone (P = 0.01). On average, luciferase activity levels were increased 1.3-fold, relative to levels in the absence of exogenous exportin 4, suggesting that exportin 4 may interact with TRα1 in ways other than simply mediating nuclear transport.

4. Discussion

Previously we showed that TRα1 participates in rapid nucleocytoplasmic shuttling. Further, our results pointed to the intriguing possibility that, in addition to following a cooperative CRM1/calreticulin-dependent export pathway during nucleocytoplasmic shuttling, TR also follows an alternative nuclear export pathway that does not rely on CRM1 (Bunn et al., 2001; Grespin et al., 2008; Mavinakere et al., 2012). Here, we propose that additional exportins play a role in modulating TR cellular localization. By coupling in vivo RNAi and FRAP experiments, we showed that knockdown of exportins 4, 5, or 7 altered nucleocytoplasmic shuttling of TRα1. In addition, overexpression of exportins 5 and 7 shifted the distribution pattern of TRα1 and TRβ1 toward a greater percentage of cells with a more cytosolic distribution. Interestingly, results differed for TRα1 and TRβ1-mediated transactivation in cells expressing exogenous exportins. When TRβ1 and exportins 4, 5, or 7 were co-expressed, there was no significant change in either repression or stimulation of T3-mediated gene expression. In contrast, when exportin 7 was co-expressed with unliganded TRα1, TRE-luciferase reporter gene activity was significantly greater compared with the control; that is, in the presence of exportin 7 unliganded TRα1 was less able to repress transcription. In addition, when exportin 4 was co-expressed with liganded TRα1, fold stimulation of the TRE-luciferase reporter gene was significantly increased compared with the control. TR subtype-specific regulation of target gene expression is not without precedent; distinct properties of the amino terminus of TRα1 have been shown to lead to 2-fold greater ligand-independent repression and ligand-dependent stimulation (Hollenberg et al., 1995). Taken together, our data provide evidence that in addition to the previously characterized role of CRM1 and calreticulin in TR export, exportins 4, 5, and 7 also influence TRα1 and TRβ1 cellular localization either directly by promoting nuclear export, or indirectly by decreasing nuclear retention. In this way, exportin levels may provide an additional level of control in modulation of the cellular response to T3.

Further investigation will be required to determine why overexpression of exportin 4 had no significant effect on TR

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**Fig. 4.** Overexpression of exportin 5 promotes nuclear export of TRα1 and TRβ1. (A) HeLa cells were transiently transfected with GFP-TRα1 expression plasmids, Myc or Myc-XPO5 (exportin 5), as indicated. Cells were immunostained with anti-Myc (red) and analyzed by fluorescence microscopy for the distribution of TRα1 (green). Nuclei were stained for DNA with DAPI (blue). Bar graph summarizes the effect of overexpressing exportin 5 on TRα1 distribution in two categories: primarily nuclear (N), or distribution patterns ranging from nuclear accumulation and a clearly visible cytosolic population to a whole cell distribution (N + C / WC). Error bars represent ± 1 SEM (n = 4 replicates each, with at least 300 cells scored per replicate). ***P < 0.001. (B) Parallel experiments were performed with HeLa cells cotransfected with GFP-TRβ1 expression plasmid and Myc or Myc-XPO5 (n = 4 replicates, 100–300 cells per replicate).
Recently, exportin 5 was shown to be important for mediating CRM1-independent nuclear export of the androgen receptor, through a NES located in the DNA binding and hinge domains of AR (Shank et al., 2008). Prior to this report, exportin 5 was only thought to be involved in regulating miRNA biogenesis by exporting the precursor miRNAs out of the nucleus (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2005). A role for exportin 5 in promoting nuclear export of TRα1 and TRβ1 further expands the potential cargo list for this versatile exportin. In addition to the cytosolic mislocalization of TR, since exportin 5 is critical for miRNA biogenesis, down-regulation of this exportin would be predicted to have widespread effects. More than 700 miRNAs have been identified in humans. With over two-thirds of protein-coding genes predicted as targets, these abundant small regulatory RNAs play important roles in modulating a broad range of cellular processes (Ebert and Sharp, 2012; Melo and Esteller, 2014). Indeed, inactivating mutations in exportin 5, and the concomitant trapping of pre-miRNA in the nucleus, are linked to human tumors with microsatellite instability, including colon, gastric, and endometrial tumors (Melo et al., 2010).

We conclude that exportin 7 is also a player in regulating shutting of TRα1 and TRβ1. Exportin 7 (also known as RanBP16) mediates export of diverse proteins with variable NESs including eIF4A1, p50RhoGAP, 14-3-3σ (Liang et al., 2011), and interacts with at least 13 downstream AMP-activated protein kinase (AMPK)-related kinases, and acts as a tumor suppressor (Dupuy et al., 2013; Gan and Li, 2014). Further, TRβ1 has been shown to increase LKB1 expression in muscle, resulting in activation of the LKB1/AMPK signaling pathway, and an increase in levels of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Branvold et al., 2008). PGC-1α enhances gene expression mediated by TR, while the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) represses gene expression. Although not linked to LKB1 levels, nuclear export of SMRT has been shown to be triggered by changes in cell activity (Guo et al., 2013; Soriano et al., 2011). It is tempting to speculate that exportin 7 promotes nuclear shuttling of proteins that modulate the LKB1 signaling axis, as it has been shown that LKB1 is a key regulator of cell growth and metabolism.

Fig. 5. Overexpression of exportin 7 promotes nuclear export of TRα1 and TRβ1. (A) HeLa cells were transiently transfected with GFP-TRα1 or GFP-TRβ1, and mCherry or mCherry-tagged exportin 4 (XPO4) expression plasmids, as indicated. Bar graph summarizes the lack of effect of exportin 4 overexpression on TR distribution in three categories: primarily nuclear (N); nuclear accumulation and a clearly visible cytosolic population (N+C); or whole cell, where the nucleus was not distinct (WC). Error bars represent ± SEM (n = 3 replicates, 100–300 cells scored per replicate). (B) Bar graph summarizing experiments performed with HeLa cells co-transfected with GFP-TRα1 or GFP-TRβ1, and HA tag or HA-tagged exportin 7 (XPO7) expression plasmids, as indicated. Error bars indicate ± SEM (n = 5 replicates, 100–300 cells per replicate). **P < 0.01.

distribution, while knockdown markedly inhibited shuttling. No cytosolic accumulation of TR was observed, suggesting that reduced recovery of fluorescence in the bleached nucleus is not due to inhibition of nuclear import. It may be that levels of endogenous exportin 4 are high enough already to be saturating for export, or exportin 4 may interact with TR in ways other than simply mediating nuclear transport. Additional roles in the cell for exportin 4 beyond transport activity are becoming better understood. A recent report implicates exportin 4 as a tumor suppressor that is down-regulated in hepatocellular carcinoma (Liang et al., 2011). In addition, exportin 4 plays a role in nuclear import of members of the Sox family of transcription factors (Gontan et al., 2009), and interacts directly with Sox 9, thereby blocking binding of Sox 9 to target genes (Tsuchiya et al., 2011). Here, we show that co-expressing exportin 4 with TRα1 increases ligand-dependent gene transactivation, but has no significant effect on ligand-independent repression. These data suggest that exportin 4 may exert its effects on TR localization indirectly, by altering nuclear retention and, thereby, promoting accessibility to the nuclear export machinery.
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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.04.014.

References


Fig. 6. Overexpression of exportins 4, 5, and 7 has variable effects on TR-mediated ligand-independent and ligand-dependent gene regulation. (A) HeLa cells were transfected with GFP-TRα1, mCherry or mCherry-exportin 4 (mCh-XPO4), Myc or Myc-exportin 5 (Myc-XPO5), or with HA or HA-exportin 7 (HA-XPO7) expression plasmids, and TRE (DR+4)-firefly luciferase reporter and Renilla luciferase internal control. Data are presented as relative ligand-independent luciferase reporter activity (firefly/Renilla) (−T3), and fold stimulation in the presence of T3. Error bars indicate ±1 SEM (n = 3 replicates of 8 wells per treatment); *P < 0.05, **P < 0.01. (B) Parallel experiments with GFP-TRβ1 (n = 4 replicates).