Characterization of the Role of the Small Ubiquitin-like Modifier (SUMO) on Nuclear Localization and Proteolysis of the Thyroid Hormone Receptor (TR)

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Characterization of the Role of the Small Ubiquitin-like Modifier (SUMO) on Nuclear Localization and Proteolysis of the Thyroid Hormone Receptor (TR)

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honors in Biology from The College of William and Mary in Virginia

By

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1 May 2017
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Abstract

The small ubiquitin-like modifier (SUMO) is involved in post-translational modification of proteins and is characterized by its role in regulation of a variety of cellular processes. The objective of this thesis was to characterize the role SUMO has in modification of the thyroid hormone receptor (TR). TR is a regulatory transcription factor that, in most cases when the ligand (T3) is not bound, represses gene expression through the recruitment of co-repressors. When T3 is bound to TR, the co-repressors are released and co-activators are recruited, resulting in positive gene expression mediated by a particular thyroid hormone response element (TRE). Previous research has identified the import and export proteins involved in nuclear localization of TR. Post-translational modification, however, has only begun to be characterized. In terms of proteolysis, TR is degraded through poly-ubiquitination; but it is unclear whether ubiquitin binding is correlated with sumoylation. SUMO sites for TR have been previously identified, and their role in TR-mediated gene expression demonstrated. Here, the role SUMO plays in nuclear localization and proteolysis of TR through its interaction with ubiquitin binding is characterized. Mutant constructs of TR that could not be sumoylated were synthesized and cloned into GFP. For nuclear localization assays, the constructs were transfected into HeLa cells and quantitatively scored for the ratio of fluorescence intensity in the nucleus versus the cytosol using region of interest (ROI) analysis. Coimmunoprecipitation followed by western analysis, was conducted in order to identify the relationship between sumoylation and ubiquitination. The nuclear localization experiments showed no changes in nuclear localization of the SUMO-deficient TR compared to the wild-type TR. Coimmunoprecipitation suggested that there may be a higher level of ubiquitination when TR was not sumoylated. However, varying levels of cellular ubiquitin make this finding inconclusive. Overall, this thesis demonstrates that SUMO is not directly involved in nuclear localization of TR; however, it may play a role in enhancing the binding of ubiquitin, ultimately suggesting that sumoylation may be involved in proteolysis of TR.

Key Words: Small ubiquitin-like modifier (SUMO), thyroid hormone receptor (TR), nuclear localization, proteolysis, ubiquitin
**Introduction**

Post-translational modification plays a critical role in protein regulation and expression throughout the body. The small ubiquitin-like modifier (SUMO) is a post-translational modifier whose binding to target proteins is shown to be involved in a range of key cellular processes (Békés and Drag, 2012). It was previously observed that SUMO is conjugated to a lysine residues on the thyroid hormone receptor (TR) (Liu et al., 2012). TR has been shown to rapidly shuttle between the nucleus and the cytoplasm adding an extra layer of regulation to this form of receptor-mediated gene expression (Bunn et al., 2001). However, the affects of post-translational modifiers on TR nuclear localization have yet to be identified. It has been shown that nuclear mislocalization is a characteristic of an oncogenic homolog of TR (Bonamy et al., 2005). Therefore, gaining a deeper understanding of what regulates the nuclear localization of TR is significant in identifying how the endocrine system mediates physiological homeostasis.

Proteolysis of TR occurs via poly-ubiquitination and proteasome-mediated degradation (Dace et al., 2000; Bondzi et al., 2011). However, previous work has identified many proteins where there is a high level of interaction between SUMO and ubiquitin when it comes to protein regulation and proteolysis (Ahner et al., 2013; Guzzo et al., 2012; Aillet et al., 2012). Therefore, it is important to identify whether the binding of SUMO correlates with the binding of ubiquitin in order to complete the picture of how TR turns-over within cells.
The Endocrine System

The endocrine system is composed of organs that release signaling molecules called hormones into the circulatory system, which are then transported to target organs where they carry out a variety of physiological effects. Endocrine signaling is relatively slow, compared to neural signaling, but long-acting. In the brain, the hypothalamus regulates the pituitary gland which ultimately controls the release of hormones from endocrine glands throughout the body. Physiologically, hormones affect a wide range of systems including the digestive, reproductive, and neurological systems, while maintaining metabolic homeostasis.

The Thyroid Gland and Thyroid Hormone

The thyroid is a butterfly-shaped gland located in the neck, below the larynx, that secretes thyroid hormone. Thyroid hormone is involved in many vital physiological processes including growth regulation, metabolic control, and central nervous system development (Visser et al., 2011). When low levels of thyroid hormone are detected, the hypothalamus releases thyrotropin-releasing hormone (TRH). TRH then stimulates the pituitary gland to release thyroid-stimulating hormone (TSH). After that, TSH stimulates the thyroid gland to produce thyroid hormone. This system is known as the hypothalamic-pituitary-thyroid axis, or the HPT axis (Figure 1). Other examples of this sort of negative feedback loop are the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis (Chiamolera and Wondisford, 2009). Thyroid hormone is secreted from follicles on the gland that are filled with colloid. The colloid contains the pro-hormone thyroglobulin that can be synthesized into thyroid
Figure 1: Overview of the HPT Axis

The hypothalamus senses low levels of T3 and T4 in the bloodstream and signals the release of thyrotropin-releasing hormone (TRH) which then stimulates the pituitary gland. Subsequently, the pituitary gland releases thyroid-stimulating hormone (TSH) which stimulates the thyroid gland ultimately leading to the release of T3 and T4. T3 and T4 enter the bloodstream and circulate to peripheral tissues. When the presence of T3 and T4 is high in the bloodstream, the hypothalamus and pituitary decrease the amount of TRH and TSH released.
hormone. There are two forms of thyroid hormone; T4 (thyroxine) and T3 (triiodothyronine)—most of which is synthesized from the deiodination of T4. T3 is the active form and can cross the blood brain barrier (St Germain et al., 2009).

**Mutations of TRA and TRB**

There are two major types of thyroid hormone receptors (TR) that have been described; TRα and TRβ. Respectively, these are encoded by the TRA gene and TRB gene. Mutations in TRA and TRB can result in thyroid-related disorders. Resistance to thyroid hormone (RTH) results from mutations in TRB and is classified by elevated levels of thyroid hormone while TSH is not suppressed (Refetoff et al., 2014). The heterozygous mutations leading to RTHβ have a dominant-negative affect resulting in the production of mutant receptors that inhibit wild-type TRβ (Cheng et al., 2010). Subsequently, this inhibition can lead to elevated levels of TRα signaling, making it difficult to identify the specific role of TRβ. Patients with a homozygous mutation for TRB exhibit features of RTHβ which include goiter, increased heart rate, and major deregulation of the HPT axis (Ferrara et al., 2012).

It has been shown that patients with TRA mutations have the potential to have growth retardation and problems with gastrointestinal function. Though TRA mutations show significant abnormalities, there is little change in actual thyroid function. There have been recent cases where patients with TRA mutations demonstrate hypothyroidism and thyroid resistance but maintain near-normal thyroid function when tested (Bochukova et al., 2012).
**Thyroid Hormone Receptor (TR)**

Thyroid hormone enters the cell through the active membrane transporter monocarboxylate transporter 8 (MCT8) where, once inside the cell, it will ultimately bind to thyroid hormone receptor (Visser et al., 2011). Inside the cell, deiodinase enzymes mediate conversion from T4 to T3 (St Germain et al., 2009). While inside the nucleus, TR acts as a transcription factor that binds to regulatory DNA sequences known as thyroid hormone response elements (TRE’s). When bound to TRE’s, TR typically forms a heterodimer with the retinoid X receptor (RXR), although TR can bind some TRE’s as a monomer or a homodimer. When T3 is not bound to TR, TR recruits a protein complex containing co-repressors to its activation function 1 (AF-1) domain—with the exception that at some negative TRE’s unliganded TR acts as an activator (Lavery and McEwan, 2005). Some examples of these co-repressors are nuclear receptor co-repressor (NCoR), silencing mediator for retinoic acid and thyroid receptors (SMRT), and histone deacetylase (HDAC). In contrast, the binding of T3 to TR results in the dissociation of the co-repressors and the recruitment of co-activators such as nuclear receptor co-activator 1 (SRC-1) (Weiss et al., 1996). Hence, the binding of T3 to TR ultimately determines the expression of certain genes through the recruitment of co-activators or co-repressors to TRE’s (Figure 2).

TR is part of a nuclear receptor superfamily, members of which are formed from a single polypeptide that folds into four main functional domains. These domains are the amino-terminal domain (A/B domain), the central DNA-binding domain (DBD), a carboxyl-terminal ligand-binding domain (LBD), and the hinge region which connects the DBD and LBD. Most nuclear receptors have a high similarity in the DBD and the
Figure 2: Thyroid Hormone Mode of Action

T3 and T4 are circulating outside the cell when they actively diffuse into the cell through monocarboxylate transporter 8 (MCT8). Once inside the cell, deiodinase enzymes facilitate the conversion of T4 to T3. T3 then enters the nucleus through the nuclear pore complex and binds to TR—though T3 could bind TR in the cytosol. Inside the nucleus, the binding of T3 to TR regulates gene expression by recruiting co-activators to thyroid hormone response elements (TRE’s).
hinge region. Nonetheless, most of the variability between receptors is found in the A/B domain and the LBD (Laudet, 1997). The AF-1 region is located in the A/B domain and its function is poorly understood (Wärnmark et al., 2003). However, it is believed to be involved in specificity of TR for certain TRE’s. Each TR isoform has a specific AF-1 domain which determines the composition of dimers formed by TR and the specificity for the DNA binding sites (Honnenberg et al., 1995).

The LBD allows the receptor to form homodimers, or heterodimers, with other members of the receptor superfamily, as well as to bind ligand (Figuiera et al., 2011). When T3 is bound to TR the activation function-2 (AF-2) region, located in the LBD, a conformational change at the position of helix 12 allows the binding of co-activators (de Arujo et al., 2010; Souza et al., 2011; Weiss et al., 1996).

**Nuclear Shuttling of TR**

Although TR regulates gene expression through binding target TREs in the nucleus, it has previously been shown that it rapidly shuttles between the nucleus and cytoplasm (Bunn et al., 2001). Nuclear import and export occurs through a complex of nucleoporin proteins known as the nuclear pore complex (NPC). A protein that shuttles through the NPC contains both a nuclear localization signal (NLS) and a nuclear export signal (NES). Proteins known as karyopherins bind to the NLS or NES of the cargo protein and mediate its import or export.

Typically, TR is localized in the nucleus at steady state; however; the nuclear localization of TR has been shown to play a key role in gene expression. For example, the mislocalization of the oncogenic homolog of TRα, v-ErbA, to the cytoplasm can
contribute to the oncogenic conversion of cells (Bunn et al., 2001; Bonamy et al., 2005; Bonamy and Allison, 2006).

Over the past decade, various factors involved with nuclear import and export of TR have been addressed. In 2003, it was shown that phosphorylation of one or more sites of TRα, not including serine 12, plays a role in either nuclear retention and/or inhibits nuclear export. However, phosphorylation is not directly involved with nuclear import (Nicoll et al., 2003). The export pathway of TRα was further characterized in 2008 when it was shown that TRα may follow a cooperative export pathway mediated by both calreticulin and the exportin CRM1 (Grespin et al., 2008).

The signals involved in nuclear export and nuclear import of TR were characterized in 2012. Here, the nuclear localization signal (NLS) for TRα and TRβ was identified in the Hinge region. Further, a second NLS was identified in the A/B domain of TRα that is not present in TRβ. This study also identified three CRM1-independent nuclear export signals (NES) in the ligand-binding domain of TR (Mavinakere et al., 2012). The exportins that correspond to these NES’ were characterized in 2015. The knockdown of exportins 4, 5, and 7 resulted in greater nuclear retention of TR demonstrating their role in nuclear export. This study also showed that overexpression of exportins 5 and 7 resulted in a shift of TR toward the cytosol (Subramanian et al., 2015). Finally, the importins involved in nuclear import of TRα were identified in 2016. This study showed that knockdown of importins 7, β1, and α1 resulted in reduced nuclear localization of TRα demonstrating their role in nuclear import and showed direct binding of these importins via coimmunoprecipitation assays (Roggero et al., 2016).
The Small Ubiquitin-Like Modifier (SUMO)

To gain a deeper understanding of what affects the nuclear dynamics of TR, the world of post-translational modification is worthy of exploration. In particular, modification by the small ubiquitin-like modifier (SUMO) is the focus of this thesis. Post-translational modification occurs when a substrate is covalently bound to an amino acid side-chain on a protein and regulates its activity after, or during, protein synthesis. SUMO is a post-translational modifier that was first identified in the 1990’s (Meluh and Koshland, 1995). Since its discovery, it has been shown that SUMO plays a role in a wide array of cellular processes including transcription, receptor function, cell cycle control, and DNA repair (Békés and Drag, 2012).

Based on primary sequence alone, SUMO and ubiquitin do not have a lot of similarity. However, they have a similar folding pattern known as the β-grasp fold, making them structurally related (Bayer et al., 1998). The mechanism and machinery used for ubiquitination and sumoylation is also very similar. SUMO and ubiquitin are covalently linked to a lysine residue on the target protein forming an amide linkage. They also both utilize E1, E2, and E3 conjugating enzymes. In the first step of sumoylation, a small peptide is removed from the C-terminus by a cysteine-specific SUMO protease called SENP in eukaryotes and ULP in prokaryotes. This exposes a di-glycine motif which interacts with the E1 enzyme. The E1 enzyme is a dimer consisting of Sae1 and Sae2. SUMO covalently attaches to a reactive cysteine residue on Sae2 through ATP-dependent thioesterification. SUMO then binds to the E2 conjugating enzyme, Ubc9, through a thioester linkage. Finally, the E3 ligase stimulates the E2 enzyme to transfer SUMO to its substrate (Gareau and Lima, 2010). Much like
ubiquitination, sumoylation is a reversible process. Desumoylation is conducted by the same proteases, SENPs/ULPs, that convert SUMO to its reactive form (Figure 3) (Mukhopadhyay and Dasso, 2007).

The function of SUMO was first characterized by Matunis et al., in 1996 as a regulator for RanGAP1. Matunis and coworkers found that for RanGAP1 to successfully be transported to the NPC it must first be modified through the binding of a “ubiquitin-like protein.” Since this initial discovery, SUMO has been identified to play a role in the nuclear transport of many proteins. In 2012 it was found that SUMO plays a role in the transport of translationally-controlled tumor protein (TCTP) into the nucleus where it functions as an antioxidant (Munirathanam and Ramaswamy, 2012). Another example is Kap114, a nuclear import receptor that carries cargo across nuclear pores and into the nucleus. It was found that for Kap114 to successfully localize to the nucleus it must be sumoylated (Rothenbusch et al., 2012).

**SUMO and Nuclear Localization of TR**

It has previously been identified that TR-dependent gene regulation requires the binding of SUMO. Both TRα and TRβ are sumoylated in vivo and in vitro—the study also suggested that the sites are likely poly-sumoylated as well. Sumoylation sites have also been identified in both TRα and TRβ. TRα is sumoylated at lysines 283 and 389 in the LBD between the two NES’. The SUMO sites for TRβ are located at lysines 50, 146, and 443 in the A/B domain, DBD, and LBD (Figure 4). Though sumoylation of TRα is not ligand dependent, TRβ will not be sumoylated if the ligand is not bound. In general, SUMO has been shown to be involved in regulation of TR-dependent gene expression
Figure 3: The Sumoylation and Desumoylation Cycle

The SUMO cycle begins when a small peptide is cleaved from the C-terminus of SUMO by SENP (ULP in prokaryotes). This exposes a di-glycine motif that binds with the E1 enzyme which is a dimer consisting of Sae1 and Sae2. SUMO then binds to the E2 enzyme Ubc9 through a thioester linkage. After that, the E3 ligase facilitates the transfer of SUMO from Ubc9 to the binding of the substrate. Finally, desumoylation occurs through the same proteases (SENP/ULP) that initially cleaved the small peptide.
The domains for TRα and TRβ are shown. There is a high degree of similarity between the domains, however TRβ does not have a second NLS in the A/B domain like TRα. Hence, the wild-type TRβ is not as localized to the nucleus compared to the wild-type TRα. TRα is sumoylated at lysines 283 and 389 in the LBD between the two NES’. The SUMO sites for TRβ are located at lysines 50, 146, and 443 in the A/B domain, DBD, and LBD (modified from Mavinakere et al., 2012).
through recruitment of various cofactors resulting in positive or negative effects depending on the TRE (Liu et al., 2012).

Though it has been shown that SUMO plays a role in TR-dependent gene expression, it is unclear whether SUMO is involved in the nuclear trafficking of TR. Based on reports in the literature of SUMO’s ability to regulate nuclear trafficking (Munirathanam and Ramaswamy, 2012; Rothenbusch et al., 2012; Anderson and Stover, 2009), the role—if any—SUMO plays in the nuclear localization of TR was investigated here. The location of the SUMO sites near an NES for TRα suggests that SUMO may play a role in its nuclear export. Hence, when TR is unable to be sumoylated, nuclear retention may be enhanced. One of the SUMO sites is also close to a known NES in the LBD of TRβ. Therefore, nuclear retention may also be increased when TRβ is unable to be sumoylated, indicating SUMO’s role in nuclear export.

**Relationship Between SUMO, Ubiquitin, and TR**

Previous studies have begun to identify the relationship between SUMO and ubiquitin. Anderson et al. (2012) characterized the interplay between SUMO and ubiquitin in terms of nuclear transport and degradation of serine hydroxymethyltransferase 1 (SHMT1). It had previously been shown that sumoylation was required for nuclear import of SHMT1 (Anderson and Stover, 2009). It was later found that the ubiquitination of SHMT1 played a role in nuclear export (Anderson et al., 2012). This suggests that competition between SUMO and ubiquitin ultimately determines the nuclear localization of SHMT1.
Because SUMO and ubiquitin both are covalently linked to lysine residues, it was initially believed that the relationship between the two was purely antagonistic (Ulrich, 2005). Therefore, the discovery of SUMO-targeted ubiquitin ligase (STUbL) proteins was very interesting because they indicated the potential for SUMO-ubiquitin conjugates. Many researchers have begun to look deeper into this “cross-talk” between SUMO and ubiquitin. There have been many recent studies demonstrating the importance of SUMO-ubiquitin chains in protein degradation. One study found that SUMO-ubiquitin chains play a role in recruitment of the proteasome for degradation of the inhibitory molecule IκBα (Aillet et al., 2012).

Another study found that SUMO-ubiquitin chains play a role in DNA repair. The breast cancer susceptibility protein (BRCA1) is targeted to double stranded breaks through poly-ubiquitination. However, it was shown that SUMO chains also play a role in targeting BRCA1 to damaged DNA for repair. With the inclusion of SUMO it was concluded the chain required for recruitment of BRCA1 must be a hybrid of SUMO and ubiquitin (Guzzo et al., 2012).

Cross-talk between SUMO and ubiquitin also has been investigated as a mechanism for the degradation of the ion channel Cystic Fibrosis Transmembrane Conductance Receptor (CFTR). It was found that for CFTR to be degraded it must be both sumoylated and ubiquitinated (Ahner et al., 2013).

In terms of the relationship between SUMO and TR, it has been confirmed that ubiquitin binds to TR for the targeting of proteasome-mediated degradation. The study showed that, like sumoylation, ubiquitination of TRβ is ligand dependent. Not only that, ubiquitination is not enough to trigger proteolysis—T3 must also be bound to TR in order
to recruit the proteasome (Dace et al., 2000). Here, we sought to identify if sumoylation
is also involved in proteolysis of TR\(\alpha\) through cross-talk with ubiquitin. Because it is
already known that poly-ubiquitination of TR\(\beta\)—not TR\(\alpha\)—is ligand-dependent, we
sought to identify what other regulatory factors are involved in the degradation of TR\(\alpha\).
Since there have not been any other regulatory factors for the degradation of TR\(\alpha\)
characterized, we presume that the binding of SUMO may be involved in proteolysis by
acting as a requirement for poly-ubiquitination. By addressing the relationship between
SUMO and ubiquitin, we were able to determine if SUMO is also a requirement for
recruitment for proteasomal-mediated degradation of TR\(\alpha\).

**Thesis Objective: Identify the role of SUMO in nuclear localization and proteolysis
of TR**

Previous research has demonstrated that the post-translational modifier SUMO is
involved in TR-mediated gene expression for both TR\(\alpha\) and TR\(\beta\) (Liu et al., 2012).
Though this work confirmed SUMO’s role in gene expression, it did not address its
involvement in nuclear localization. It has been shown that TR rapidly shuttles between
the nucleus and cytoplasm (Bunn et al., 2001), leading to investigations of the
localization signals and karyopherins involved in nuclear localization of TR (Mavinakere
et al., 2012; Subramanian et al., 2015; Roggero et al., 2016). Thus, uncovering the role
post-translational modification plays in nuclear localization is the next step in uncovering
what regulates the nuclear to cytoplasmic shuttling of TR. Since the post-translational
modifier of interest here is SUMO, the first objective of this thesis was to identify
whether SUMO plays a role in nuclear localization of both TR\(\alpha\) and TR\(\beta\).
Secondly, previous studies have demonstrated that there is a significant relationship between SUMO and ubiquitin. In many cases, there is a fair amount of “cross-talk” between the two proteins adding an extra level of regulation to their activity (Ahner et al., 2013; Guzzo et al., 2012; Aillet et al., 2012). It has already been identified that poly-ubiquitination is a trigger for the proteolysis of TR (Dace et al., 2000). However, this does not address whether or not ubiquitination is dependent on SUMO. Overall, if a mutant receptor is unable to be degraded properly then it will accumulate in the cell which would ultimately be detrimental to the gene expression it regulates. Gaining a complete picture of how TR is degraded is significant in understanding what regulates receptor proteolysis in the endocrine system. Therefore, the second objective of this thesis was to identify the relationship between the binding of SUMO and ubiquitin to TR.

**Materials and Methods**

**Plasmids**

The SUMO sites for TRα and TRβ were modified at their respective positions. Hence, the lysine residues were changed to glutamine at positions 283 and 389 for TRα, and 50, 146, and 443 for TRβ. Constructs were designed using GeneArt gene synthesis services (Life Technologies). The GeneArt parent vectors were digested with restriction endonucleases—Bgl II and Kpn I for TRα, and BamH I and Kpn I for TRβ—to cut out the TR cDNA insert and then purified using a DNA purification kit (Qiagen). The inserts were separated from the vectors by size on a 1.5% agarose gel and visualized by staining with ethidium bromide. A gel slice containing the insert band was purified using a gel
extraction kid (Qiagen) and ligated into a GFP expression vector. The new GFP-tagged TR expression plasmids were sequenced in-house to confirm the success of the ligation. The ligated plasmids were transformed into 5-alpha competent E. coli cells (NEB) and then isolated using a plasmid midiprep kit (Zymo Resesarch).

**Cell Culture and Transfection**

HeLa cells were grown at 37°C in Minimal Essential Media (Gibco) with an addition of 10% fetal bovine serum. For cell scoring, HeLa cells were seeded on glass coverslips at 2.5-3.0 x 10^5 cells/mL in 6-well plates. Cells were transfected 24 hours after seeding with 2 µg of plasmid DNA and 4 µg of Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). Opti-MEM was replaced with Minimal Essential Media 5 hours after transfection. Twenty four hours post-transfection, slides were washed in D-PBS, fixed using 3.7% formaldehyde, and mounted on microscope slides with Fluoro-Gel II containing DAPI (Electron Microscopy Science).

For GFP-Trap coimmunoprecipitation assays, cells were seeded at 11 x 10^5 cells/mL in a 100 mm vented dish. Twenty four hours after seeding, cells were transfected with 10 µg of plasmid DNA (5 µg of HA-tagged Ubiquitin expression plasmid + 5 µg of the respective GFP-construct) and 20 µg of Lipofectamine 2000 in Opti-MEM. Opti-MEM was replaced with Minimal Essential Media 8 hours after transfection.
**Mean Fluorescence Scoring**

Slides were blinded prior to scoring and 100 cells per replicate were scored by obtaining the mean fluorescence of a region of interest (ROI) in both the nucleus and the cytoplasm. Blinding ensured that there was no bias from knowing the plasmids from each slide prior to scoring. Nikon NIS Elements software was used to quantify the ROI value and transfer to Microsoft Excel where the N/C ratio was calculated and normalized to the wild-type. In terms of un-normalized data, an N/C ratio of 1.0 represents a “whole-cell” distribution pattern. Thus, a ratio higher than 1.0 means that the distribution is shifted towards the nucleus. Student t-tests were conducted to note any changes in localization pattern of statistical significance. If a cell contained aggregates it was ensured that the selected ROI did not involve the aggregate. Consequently, the distribution patterns of the aggregates were noted through semi-quantitative analysis.

**Semi-Quantitative Scoring of Aggregation Patterns**

Patterns of aggregation were simultaneously acquired while mean fluorescence was obtained. 100 cells were scored, and if a cell contained aggregates the distribution pattern was noted. On average, about 40% of the scored cells contained aggregates. The three categories for distribution were aggregates localized to the nucleus, cytoplasm, or throughout the whole-cell. The average number of cells with each distribution pattern was taken after all the replicates were obtained.
**GFP-Trap Coimmunoprecipitation Assays**

Twenty-six hours after transfection cells were trypsinized and collected, washed three times in D-PBS, and lysed (Chromotek). Lysates were tumbled in GFP-Trap Agarose Beads (Chromotek) at 4°C for 2-3 hours. Beads were separated through centrifugation and the supernatant was collected to identify “unbound” protein patterns. The “bound” samples, or the beads, were washed in a 1% BSA Tris-buffered Saline (TBS) plus 0.1% Tween solution and re-suspended in 2X SDS-PAGE Sample Buffer for SDS-PAGE.

**SDS-Page and Western Blot**

20 µl of each immunoprecipitation sample were separated using SDS-PAGE on an 8% gel. Protein size was detected using Kaleidoscope Protein Standards (Bio-Rad). Proteins were transferred to a PVDF membrane using the iBlot 7-minute Blotting System (Invitrogen). Membranes were blocked overnight by shaking at 4°C in a solution of TBS and blocking agent (GE Healthcare). The next day membranes were washed in TBS and incubated with the primary antibody for 2 hours at room temperature. The solution for the primary antibody contained TBS, blocking agent, and 1:4,500 dilution (µg/mL) of anti-HA antibody (to detect HA-tagged ubiquitin). The primary antibody was washed off with TBS for an hour and then the secondary antibody was added and incubated for 1.5 hours at room temperature. The secondary antibody solution contained TBS, blocking agent, and a 1:25,000 dilution (µg/mL) of horseradish peroxidase-conjugated goat anti-rabbit (GE Healthcare). The secondary antibody was washed for an hour and the blots...
underwent chemiluminescent detection using SuperSignal West Femto Stable Peroxide Buffer and Enhancer Solution (Thermo Scientific) and exposed to X-Ray film.

**Results**

**Sumoylation of TRα does not affect nuclear localization**

To determine whether sumoylation plays a regulatory role in nuclear localization of TRα, an expression plasmid for GFP-tagged mutant TRα was designed encoding altered residues at the sites for sumoylation; positions 283 and 389 located in the ligand binding domain (LBD) (Liu et al., 2012). Changing the residues from lysine to glutamine, generated a mutant TRα that would be unable to be sumoylated. After transient transfection into HeLa cells, the intracellular distribution of the mutant construct was compared to the wild-type GFP-TRα and an empty-GFP vector using fluorescence microscopy. In general, GFP-TRα remained mainly nuclear, as expected (Bunn et al., 2001), while the empty-GFP had more of an even distribution between the nucleus and cytoplasm.

Upon initial observation, there was no apparent difference in nuclear distribution of the mutant of TRα compared with wild-type TRα (Figure: 6). The nuclear/cytosolic (N/C) ratio was calculated using mean fluorescence scoring and each replicate (N=3, 100 cells scored per replicated) was normalized so that the wild-type GFP-TRα had an N/C ratio of 1.0 (Figure: 5). The average of the normalized N/C ratios of the SUMO mutant was 1.02+/− 0.09. There was no significant difference between the mutant and the wild-type (p=0.872), which matches the qualitative observation that there was essentially no shift in nuclear localization when the SUMO sites were modified. In comparison, the
normalized N/C average for the empty-GFP vector was 0.55+/- 0.08 which was significantly different from wild-type TRα (p=0.010). Thus, a mutant of TRα that cannot be sumoylated ultimately does not have altered nuclear localization when compared to the wild-type, suggesting that SUMO modification does not play an important role in nuclear localization.

**Sumoylation of TRβ does not affect nuclear localization or alter aggregate formation**

Given that the SUMO sites have previously been identified for TRβ (Liu et al., 2012) the role SUMO plays in the nuclear localization of TRβ is now of interest. The sumoylation sites fall in a different region of TRβ compared with TRα, therefore it is of interest to determine whether SUMO modification plays a role in nuclear localization of this TR subtype. To this end, a mutant expression plasmid for GFP-tagged TRβ that cannot be sumoylated was constructed. The lysines at residues 50, 146, 443 were modified to glutamines effectively knocking out the SUMO sites for TRβ. After transient transfection into HeLa cells, the intracellular distribution of the mutant was compared to wild-type GFP-TRβ and an empty-GFP vector. Similarly to TRα, there was no apparent shift in nuclear localization upon initial observation by fluorescence microscopy (Figure: 8). However, as expected TRβ has much more of a cytosolic distribution compared to TRα (Mavinakere et al., 2012). The N/C ratios of each replicate (N=3, 100 cells scored) were normalized so that the wild type is 1.0 (Figure: 7). The average normalized ratio for the mutant of TRβ was 0.99+/- 0.03. There was no significant difference between the wild-type and the mutant (p=0.872). In contrast, the average normalized N/C ratio of the
empty-GFP vector was 0.75±0.04. The difference between GFP-TRβ and the empty-GFP vector was statistically significant (p=0.006). These findings indicate that SUMO modification does not play an essential role in nuclear localization of TRβ.

It was also observed that a portion of the GFP-TRβ cells contained aggregates. At this point it is unclear as to why GFP-TRβ develops aggregates, but it could be due to the nature of the folding pattern of the plasmid when tagged with GFP. To determine if a SUMO mutant of TRβ modifies its pattern of aggregation, the aggregates of TRβ were scored semi-quantitatively after transient transfection into HeLa cells. Cells were divided into three categories based on the patterns of their aggregates; cells where the aggregates were mainly located in the nucleus, located in the cytosol, or were evenly distributed throughout the whole-cell. The average number of aggregates for each category demonstrated a similar pattern of aggregation between the wild-type and mutant TRβ (Table: 1) (Figure: 9). In general, the marginal differences between the aggregation patterns provide evidence that SUMO does not alter aggregate formation in TRβ.

The sumoylation mutant of TRα may have a higher level of ubiquitination than wild-type TRα

Previously, the relationship between SUMO and ubiquitin has been characterized in other proteins (Ahner et al., 2013; Guzzo et al., 2012; Aillet et al., 2012). However, the relationship between SUMO and ubiquitin has not been characterized for TRα. HeLa cells were co-transfected with expression plasmids for the mutant of GFP-TRα that cannot be sumoylated while ubiquitin was over-expressed. Subsequently, cells were lysed and “GFP-trap” coimmunoprecipitation assays were conducted to identify any
relationship between sumoylation and ubiquitination. Protein patterns of the mutant were compared to wild-type TRα and revealed on western blots (Figure: 10). The overall pattern of the western blots suggested an increase in ubiquitination of SUMO-deficient TRα (N=9). However, there were some replicates where there was a higher level of ubiquitin when TRα was sumoylated (N=3), and a single replicate that had an even amount of ubiquitin between the mutant and wild-type TRα.

Results are inconclusive because there was a high-level of inconsistency in ubiquitin levels in the supernatant from each trial. This suggests that, in general, the levels of ubiquitin vary widely between cells. Although it is tempting to conclude that the sumoylation mutant of TRα has a higher level of ubiquitination than wild-type TRα, no conclusion can be confirmed at this time, since these results could be due to the varying ubiquitin levels within cell populations.
**Figure 5:** Mean fluorescent values of SUMO-deficient mutant of TRα do not demonstrate a change in nuclear localization when compared to the wild-type

Nuclear/Cytosolic (N/C) ratio calculated from mean ROI intensity of transfected cells. The N/C ratio of each cell was calculated and 100 cells were scored for each replicate. For each replicate, ratios were normalized so that the wild-type GFP-TRα=1.0. The average, normalized, N/C ratio was calculated after multiple trials (N=3). Error bars represent +/- SEM.
Figure 6: Patterns of Fluorescence of Transfected HeLa Cells with Constructs of TRα

Empty-GFP displayed a whole-cell distribution. Conversely, the wild-type TRα and the SUMO-deficient mutant of TRα were significantly more localized to the nucleus.
Figure 7: Mean fluorescent values of SUMO-deficient TRβ do not demonstrate a change in nuclear localization when compared to the wild-type

Nuclear/Cytosolic (N/C) ratio calculated from mean ROI intensity of transfected cells. The N/C ratio of each cell was calculated and 100 cells were scored for each replicate. For each replicate, ratios were normalized so that the wild-type GFP-TRβ=1.0. The average, normalized, N/C ratio was calculated after multiple trials (N=3). Error bars represent +/- SEM.
Empty-GFP had a whole-cell distribution. The wild-type for TRβ and the SUMO-deficient mutant of TRβ had some cytosolic localization; however, there was a higher amount of localization in the nucleus.
Table 1. Average number of cells containing aggregates in varying distribution patterns in TRβ

<table>
<thead>
<tr>
<th></th>
<th>Whole-Cell</th>
<th>Nuclear</th>
<th>Cytosolic</th>
<th>Total Number of Cells with Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>empty-GFP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP-TRβ</td>
<td>7.67</td>
<td>27.33</td>
<td>8.67</td>
<td>43.67</td>
</tr>
<tr>
<td>SUMO-deficient TRβ</td>
<td>8.67</td>
<td>27.00</td>
<td>7.33</td>
<td>43.00</td>
</tr>
</tbody>
</table>

Averages come from 3 replicates where 100 cells were scored per replicate. The average number of cells containing aggregates were scored based on where the aggregates were primarily localized in each cell; the nucleus, the cytoplasm, or throughout the whole cell.
Aggregation patterns of cells found in constructs of TRβ were semi-quantitatively scored into three categories: cells where the aggregates were mainly nuclear, cytosolic, or with a more whole-cell distribution. 100 cells were scored per replicate and aggregates were found in <50% of the cells scored. Averages of each category (see Table 1) were taken after three trials (N=3). Error bars represent +/- SEM.

Figure 9: Semi-quantitative analysis demonstrates no changes in patterns of aggregation in SUMO-deficient TRβ compared to wild-type TRβ
Figure 10: Examination of interplay between SUMO and ubiquitin

GFP-Trap coimmunoprecipitation assays were conducted in order to determine the relationship between SUMO and ubiquitin binding. The “bound” lanes denote the levels of ubiquitin bound to GFP-TRα, while the “unbound” lanes denote the amount of ubiquitin that was not bound to GFP-TRα. The blot above represents the general finding of this assay—there appeared to be a higher level of ubiquitin bound to TRα when it is unable to bind SUMO. However, the inconsistent levels of ubiquitin in the unbound supernatant suggests that this finding could be the result of varying levels of cellular ubiquitin.
Discussion

Liu et al. (2012) identified the sites that are sumoylated on both TRα and TRβ, while also demonstrating that SUMO plays a role in TR-dependent gene regulation. Though SUMO is involved in the recruitment of various co-factors to TREs to ensure positive or negative gene expression, prior to this thesis research the role SUMO plays in nuclear localization of TR had yet to be investigated. Because there are SUMO sites located near NES’ for both TRα and TRβ, it was hypothesized that SUMO may be involved in nuclear export or nuclear retention. However, when a mutant of TR that cannot be sumoylated was transfected into HeLa cells and scored quantitatively, no changes in nuclear localization for either TRα nor TRβ were apparent. This suggests that SUMO does not play a key role in nuclear localization of TR.

The relationship between SUMO and ubiquitin has been characterized previously. Dace et al. (2000) showed that proteolysis is triggered by poly-ubiquitination for both TRα and TRβ. The team also found that proteolysis is ligand dependent for TRα and TRβ, while the binding of ubiquitin is ligand-dependent for TRβ as well. However, the regulatory factors—besides the ligand—involved in the proteolysis of TRα have not been identified. Based on coimmunoprecipitation assays, this thesis suggests that there is a higher level of ubiquitination when TRα is not sumoylated. Liu et al. (2012) demonstrated that sumoylation of TRα is not ligand-dependent. Therefore, it is a possibility that the removal of SUMO can result in poly-ubiquitination which will ultimately lead to proteolysis. The results from the coimmunoprecipitation assay presented here are not unequivocal—potentially due to varying levels of ubiquitin within the cell. Thus, more experiments must be conducted in order to confirm this finding.
That being said, the results do suggest that there is a potential for SUMO to play a role in the regulation of proteolysis of TRα.

**Future Directions and Conclusions**

Though this thesis suggests that SUMO does not play a role in the nuclear localization of TRα or TRβ, this finding needs to be confirmed for TRα. Due to the fact that TRβ has a slightly more whole-cell distribution (Mavinakere et al., 2012), nuclear shifts, especially when looking at reduced nuclear export or enhanced nuclear retention, would be more prominent. Conversely, TRα is primarily nuclear to begin with making changes involved with reduced nuclear export less noticeable (Bunn et al., 2001). Therefore, more assays need to be conducted to confirm that SUMO does not play a role in the export of TRα. Fluorescence recovery after photobleaching (FRAP) analysis can be conducted to identify intranuclear mobility and trafficking patterns of TR between the nucleus and cytosol. If it is demonstrated that SUMO does not play a role in the kinetics of TRα movement, it can be confirmed that SUMO is not involved in nuclear localization or intranuclear mobility.

Another aspect that can be characterized is the role of the ligand on nuclear localization—particularly for TRβ. It has previously been shown that the ligand must be bound to TRβ in order for it to be sumoylated, therefore the binding of the ligand may play a role in the binding of other proteins necessary for nuclear localization (Liu et al., 2012). Thus, studies that elucidate the role T3 has on nuclear localization, or on other proteins involved in nuclear localization, would contribute to the body of knowledge regarding nuclear localization of TR.
Though it is already known that the phosphorylation of TR is involved in nuclear retention (Nicoll et al., 2003), there are other post-translational modifications that may play a role in the nuclear dynamics of TR. Besides SUMO, sites for acetylation have also been identified (Sanchez-Pacheco et al., 2009). Since this identification, work has been done that has shown that acetylation does modify the nuclear dynamics of TR. When mutants of TR were developed that either mimicked acetylation or could not be acetylated, results demonstrated that the acetylation-mimic had reduced nuclear localization. This demonstrates that acetylation plays a role in the regulation of the nuclear localization of TR (Anyetei-Anum, 2016; Anyetei-Anum, Unpublished Data). Naturally, the next step in uncovering the role acetylation plays in nuclear localization of TR would be to determine whether its role is to inhibit import or facilitate export.

In order to identify the role SUMO plays in the proteolysis of TR, further experimentation must take place. This thesis suggests that SUMO regulates ubiquitin binding when it is removed. However, the coimmunoprecipitation assay conducted here yielded inconsistent protein patterns of ubiquitination meaning that these results are inconclusive. In order to confirm these results, this assay can be further modified and optimized.

If it is confirmed that the removal of SUMO from TR is a trigger for poly-ubiquitination, there are many more questions as to how this regulation occurs. One explanation would be that the removal of SUMO results in a conformational change to TRα which would increase the affinity for ubiquitin binding and result in poly-ubiquitination. Another explanation would be that the lysines SUMO binds to are also sites for poly-ubiquitination, meaning that the removal of SUMO un-blocks the site
allowing ubiquitin to bind (Anderson et al., 2012). Due to the fact that there is a higher amount of ubiquitin binding when SUMO is not bound to TRα, it is unlikely that SUMO-ubiquitin chains are involved in proteolysis.

In order to begin answering these questions, the ubiquitin sites of TR must first be obtained. Currently, the lysine residues subject to ubiquitination of TR are unknown. If ubiquitin binds to TRα at the same residue as SUMO, then it is likely that SUMO blocks TRα from poly-ubiquitination. Hence, the removal of SUMO would trigger poly-ubiquitination and ultimately degradation. If ubiquitin does not bind to the same lysine residue as SUMO, then it is likely that the removal of SUMO would result in a conformational change to TRα allowing poly-ubiquitination to occur.

Future experimentation can also take place to identify the role SUMO plays in the proteolysis of TRβ. Currently, the proteolysis of TRβ is highly regulated in that its ligand-dependence is two-fold. The ligand must be bound to TRβ for ubiquitin to bind and for proteolysis to be triggered (Dace et al., 2000). Liu et al. (2012) also demonstrated that the binding of SUMO to TRβ is ligand-dependent, suggesting that SUMO may also be a part of the cascade of events that triggers the proteolysis of TRβ. Therefore, a coimmunoprecipitation assay, similar to the one conducted here for TRα, would be beneficial for understanding TRβ regulation, by demonstrating whether the binding of SUMO is necessary for the binding of ubiquitin.

Another avenue that can be explored is the potential cross-talk between SUMO and phosphorylation. One lab found that SUMO conjugation can be dependent on phosphorylation (Mohideen et al., 2009), indicating that its cross-talk may not be limited to ubiquitin. Another example of the ability for SUMO to regulate phosphorylation is
through modification of the α subunit of casein kinase II resulting in altered phosphorylation patterns of its substrates (Yao et al., 2011). Since it is already known that phosphorylation of SUMO is involved in nuclear localization of TR (Nicoll et al., 2003), the interaction between SUMO and phosphate could be an interesting investigation.

This thesis concludes that SUMO is not directly involved in the nuclear localization of TR. Though this can be confirmed with TRβ, further experimentation is needed to confirm that SUMO is not involved in the export or nuclear retention of TRα. Secondly, this thesis suggests that SUMO may play a role in the proteolysis of TRα through regulating the binding of ubiquitin. However, this result needs future experimentation to be confirmed. Understanding what regulates the nuclear dynamics and degradation of TR is essential in understanding TR’s role physiologically. Mis-localization of TR is associated with disease that includes cancer and metabolic dysfunction (Bonamy et al., 2005; Bunn et al., 2001; Mavinakere et al., 2012). Not only that, but the ability for degradation to occur properly is a necessity in maintaining homeostasis of the cell. If TR is unable to be properly degraded, mutated receptors would accumulate which would be detrimental to the cell and the genes that TR regulates. Overall, this research contributes to the understanding of how SUMO regulates TR which adds to the body of knowledge of receptor-mediated gene expression. A deeper understanding of what regulates TR ultimately leads to higher understanding of how the endocrine system functions and physiological regulation of the human body.
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