Mutant Thyroid Hormone Receptors and the Potential Effects on Cancer

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Abstract

Thyroid hormone receptor α1 (TRα1) mediates the expression of thyroid hormone-responsive genes, and is vital for normal human metabolic function and development. Wild-type TRα1 resides primarily in the nucleus, but undergoes rapid shuttling between the nucleus and cytoplasm. Mutated TRα1 has been found in a number of different cancers, including renal clear cell carcinoma (rc) and thyroid papillary carcinoma (tc). To test the hypothesis that receptor mislocalization is associated with oncogenesis, the intracellular distribution patterns of two cancer-associated mutants were characterized by transient transfection in HeLa cells (human cells) of expression plasmids for fluorescent protein-tagged rc6-TRα1 (I116N, A225T, M388I) and tc15-TRα1 (S183N, H184Q, R228H). rc6-TRα1 was found to have a significantly increased cytoplasmic localization compared to wild-type TRα1, and a phenotype characterized by aggregate formation. In cotransfection assays, rc6-TRα1 also colocalized with v-ErbA, a highly mutated, retroviral oncogenic form of TR. rc6-TRα1 was also found to colocalize with GFP-170 and GFP-250, bona fide markers for nuclear and cytoplasmic aggresomes, respectively. Aggresomes are highly organized protein aggregates that serve as a defense mechanism from cellular stress, in order to minimize the damage of misfolded proteins. Taken together, these results suggest that rc6-TRα1 may follow similar cellular pathways to v-ErbA and have similar effects on the cell. tc15-TRα1 was found to have no significant shift in nucleocytoplasmic localization and lacked aggregate and aggresome formation, suggesting an alternate pathway by which this mutant contributes to oncogenesis.
Contents

1. Introduction 1

1.1 Thyroid Hormone Receptor 3
  1.1.1 Structure 4
  1.1.2 Synthesis and Pathway of Thyroid Hormone 5
1.2 Protein Folding 6
  1.2.1 Aggresomes 7
  1.2.2 Chimeric Protein Markers 8
1.3 Nucleocytoplasmic Transport 8
  1.3.1 Nuclear Localization Signals and Nuclear Export Signals 9
  1.3.2 Nuclear Pore Complexes 10
  1.3.3 General Nuclear Import and Export Pathways of TRα1 11
1.4 Specific Aims of Thesis 16

2. Methods 16

  2.1 Fluorescent Fusion Protein Constructs 16
  2.2 Plasmid Preparation 17
  2.3 Tissue Culture 17
  2.4 Seeding and Transfection 18
  2.5 Fixation and Staining of Slides 18
  2.6 Fluorescence Microscopy and Scoring Slides 18
  2.7 Confocal Microscopy 19
  2.8 Colocalization Analysis 20

3. Results 21

  3.1 rc6-TRα1 has a significantly greater cytoplasmic localization than wild-type TRα1 21
  3.2 rc6-TRα1 colocalizes with v-ErbA 24
  3.3 rc6-TRα1 colocalizes with aggresomal markers GFP-250 and GFP-170 25
  3.4 rc6-TRα1 single mutation phenotypes have wild-type N/C ratios 26

4. Discussion 29

  4.1 Summary 29
  4.2 Aggresomes: A Pathway to Oncogenesis? 30
  4.3 Future Directions 32

5. Appendix 32

6. References 34
1. Introduction

Thyroid hormone is crucial for systemic metabolic regulation, central nervous system development, and lipid metabolism. The thyroid gland releases thyroid hormone in an active form and inactive form, T3 and T4, respectively. Thyroid hormone travels through the bloodstream to target tissues. After entering cells through facilitated diffusion, thyroid hormone binds to thyroid hormone receptor (TR) in the cytoplasm or in the nucleus (Henneman et al., 2001).

There are two major subtypes of TR, α and β (Lazar, 1993). In the absence of hormone, TR binds DNA response elements in several distinct pathways (Tsai, 1994). Positive response genes are repressed by unbound TR but upregulated when TR is bound by thyroid hormone. Negative response genes are upregulated by TR but are repressed when TR is bound by thyroid hormone. Additionally, TR can bind corepressors and coactivators in order to effect gene expression in a hormone independent manner. TR is also known in some contexts to be a tumor suppressor (Tsai, 1994).

TR is found primarily in the nucleus but is also present in the cytoplasm. Nuclear shuttling is the process by which macromolecules cross the nuclear envelope in order to enter or exit the nucleus. TR has multiple nuclear localization signals (NLSs) which bind importins (Mavinakere et al., 2012). Importins are large proteins that can interact with and pass through nuclear pore complexes (NPCs). The NPC acts as a selective portal for transporting large biological substrates between the nucleoplasm and the cytoplasm through the nuclear envelope (Hoelz et al., 2015). TR also has multiple nuclear export signals (NESs) that bind exportins and allow for TR to pass through the NPC into the cytoplasm (Mavinakere et al., 2012). The
competition between the binding rates of NLS to importins, TR to DNA, and NES to exportins effects the localization of TR within the cell. Increased localization of TR in the cytoplasm, termed mislocalization, may contribute to the oncogenesis of certain cancers due to the misregulation of vital regulatory genes (Bondzi et al., 2011).

This thesis discusses the characterization of two mutant forms of TRα1 associated with cancer. Cancer is a type of abnormal cell growth in which cell division becomes unregulated. Cancerous cells have a number of different gene mutations which contribute to cell cycle disregulation and which allow them to escape conventional means of cell death like apoptosis or immune system responses.

The first TRα1 mutant investigated in this thesis has three amino acid substitutions: Isoleucine-116-Asparagine, Alanine-225-Threonine, Methionine-388-Isoleucine, referred to as rc6-TRα1. rc6-TRα1 was first identified in patients with renal clear cell carcinoma, a type of kidney cancer (Rosen et al., 2011). Two of the mutations in rc6-TRα1 are in the ligand binding domain, LBD (A225T, M288I), and the other mutation is in the DNA binding domain, DBD (I116R) (Rosen et al., 2011). Within the LBD there are multiple NESs, which allow TR to exit the nucleus (Mavinakere et al., 2012). rc6-TRα1 is thus of particular interest because of its mutations in and around the LBD, and the potential effects on localization that these mutations could confer. rc6-TRα1 also has a dominant negative phenotype, a possible factor in the pathogenicity of the mutant (Rosen et al., 2011). The research presented in this thesis is novel in many aspects because rc6-TRα1 has not been fully characterized. The work presented here will help define changes in intracellular localization patterns of the rc6 mutant and how the cell is impacted by these changes.
The second mutant investigated in this thesis for altered intracellular localization patterns is tc15-TRα1, a mutant TRα1 found in thyroid papillary carcinomas. tc15-TRα1 has three mutations: Serine-183-Asparagine, Histidine-184-Glutamine, Arginine-228-Histidine (Puzianowska-Kuznicka et al., 2002). S183N and H184Q both occur in the hinge domain, while R228H occurs in the LBD. tc15-TRα1 exhibits dominant negative activity as well as lowered transcriptional activation.

The viral oncoprotein form of TRα1, v-ErbA, is known to associate with aggresomes (Bondzi et al., 2010). Aggresomes are highly organized protein aggregates that serve as a defense mechanism from cellular stress in order to minimize the damage of misfolded proteins (Bondzi et al., 2010). Additionally, v-ErbA has dominant negative activity due to the dimerization of v-ErbA and wild-type TRα1. This thesis investigates whether rc6-TRα1 and tc15-TRα1 also are recruited to aggresomes, and discusses how this process may lead to TR mislocalization.

1.1 Thyroid Hormone Receptor

TR is a part of the nuclear receptor superfamily (McKenna et al., 1999). This family includes receptors for the steroid hormones, such as androgen, estrogen, glucocorticoid, progesterone, and mineralocorticoid. This group is referred to as type I receptors. Type I receptors, the steroid receptors, are sequestered by heat shock proteins when not bound to ligand thus preventing them from affecting their cognate promoters. Also included in the nuclear receptor superfamily are the type II receptors for thyroid hormone, retinoic acid (both trans and cis forms), and vitamin D. Type II receptors bind to response elements with direct repeats, as either homodimers or heterodimers, and can bind DNA without a ligand present. TR specifically forms both a low
affinity homodimer and a high affinity heterodimer with 9-cis retinoic acid receptor (Lazar, 1993). Type III receptors have an unknown ligand and are referred to as orphan receptors.

1.1.1 Structure

Figure 1. Structures of the domains for TRα1, rc6-TRα1, tc15-TRα1, and v-ErbA. NLS and NES locations within the domains of rc6-TRα1 and tc15-TRα1 are identical to TRα1.

Nuclear receptors have multiple, conserved domains (Tsai et al., 1994) (Figure 1). In TR, the domain at the N-terminal, called the A/B domain, is highly variable in both sequence and length (Kumar et al., 2006). Within the A/B domain often lies a transactivation function that affects target genes through coactivators, corepressors, or other transcriptional machinery. The A/B domain of TRα1 also contains an NLS, referred to as NLS-2 that is absent in TRβ1 (Mavinakere et al., 2012). The C domain, or DNA binding domain (DBD), recognizes and binds response elements via two zinc finger motifs (Tsai et al., 1994). The DBD is also where dimerization occurs. The D domain, called the hinge domain, is a flexible region which connects the C
domain to the E domain. Both isoforms of TR have NLS-1 in the hinge domain. The E domain is known as the ligand binding domain (LBD). The LBD contains three NESs: NES-H3/NES-H6 and NES-H12. NES-H3/NES-H6 have overlapping sequences but are within Helices 3 and 6 respectively. The F domain has no known function. TRα1 is the only TR subtype which has an F region.

Figure 1 also shows the locations of amino acid substitutions in the two cancer-associated mutants that are the focus of this thesis. rc6-TRα1 has two mutations in the LBD and one in the DBD (Rosen et al., 2011). I116N lies within the DBD, while A225T and M388I are within the LBD. A225T is also in NES-H3/H6. tc15-TRα1 has two mutations in the hinge domain and one in the DBD (Puzianowska-Kuznicka, 2002). S183N and H184Q lie within the hinge domain, while R228H lies within the LBD, specifically within NES-H3/H6. The impacts of these mutations are investigated in this thesis.

The structure of the viral oncoprotein v-ErbA differs from TRα1 by the presence of a retroviral gag sequence fused to the A/B domain (Bondzi et al., 2011). v-ErbA has several point mutations and deletions which dramatically affect phenotype. Of consequence is the point mutation within the NLS motif of NLS-2 which renders this NLS nonfunctional. Furthermore, there is a strong NES in the gag sequence which contributes to the high cytosolic localization of v-ErbA compared to TRα1.

1.1.2 Synthesis and Pathway of Thyroid Hormone

Thyroid hormone (TH) is regulated by a negative feedback loop along the hypothalamus-pituitary-thyroid (HPT) axis (Yen, 2001). Thyrotropin releasing hormone (TRH) is produced in the hypothalamus and transported via axons to the anterior pituitary gland. TRH binds to TRH
receptors within the anterior pituitary gland thus triggering the production and release of thyroid stimulating hormone (TSH). TSH travels through the bloodstream to the thyroid gland and binds TSH receptors thus causing the synthesis and release of TH.

TH is released by the thyroid gland in two forms: 3,5,3’,5’-tetraiodo-L-thyronine (T4) and 3,5,3’-triiodo-L-thyronine (T3) (Yen, 2001). T4 is the primary form of TH released from the thyroid. Most T3 is created through the deiodination of T4 by Type I deiodinase and Type II deiodinase. Type I deiodinase is found in the liver and kidneys and releases most of the T3 synthesized back into circulation. Type II deiodinase is found in the brain, pituitary gland, and in brown adipose tissues where synthesis of T3 occurs for intercellular use. Most T4 and T3 is bound to one of several carrier proteins, but only unbound TH can enter cells and elicit a response. T3 has a higher binding affinity for TR.

1.2 Protein Folding

Proteins are formed from sequences of amino acids joined by peptide bonds. Proteins fold into a highly stable, specific native conformation. The native conformation usually sequesters hydrophobic amino acids towards the center while allowing hydrophilic amino acids to interact with water molecules in the surroundings. This process is known as the hydrophobic effect and is driven by entropy. The hydrophobic effect is the primary force that causes tertiary and quaternary protein folding (Southall et al., 2001). Proteins with mutations that expose hydrophobic amino acids to the surroundings may change conformation in order to become more stable or they may aggregate together (Johnston et al., 1998).

Occasionally, proteins misfold in such a manner that they cannot be refolded into their native state. Cellular responses include the activation of heat shock proteins (Hsp) and the poly-
ubiquitination of the misfolded protein that ultimately culminates in protein degradation via the proteasome (Goldberg, 2003). Protein aggregates can also be degraded in a similar fashion. The proteasome degradation pathway is highly accurate and efficient. The effects of protein aggregation on the localization of TRα1 mutants will be discussed in this thesis.

1.2.1 Aggresomes

Cytosolic aggresomes are clusters of perinuclear, protein aggregates that form around the microtubule organizing center (MTOC), specifically the centrosome, and are ensnared by a web of the intermediate filament, vimentin (Johnston et al., 1998). Cytosolic aggresomes recruit molecular chaperones like Hsp70 and Hsc70 which bind to exposed hydrophobic regions in order to minimize aggregation (Fu et al., 2005; Johnston et al., 1998). Aggresome formation is a cellular stress response that occurs when the production of aggregate-prone proteins exceeds the rate of degradation via the proteasome. Nuclear aggresomes are organized similarly to cytosolic aggresomes. Nuclear aggresomes also form vimentin cages, recruit molecular chaperones, and recruit proteasomes. Hsc70 is not, however, found in nuclear aggresomes. Nuclear aggresomes sometimes take on a ribbon-like structure as opposed to the rounded shape of cytosolic aggresomes.

Aggresomes, both nuclear and cytosolic, are dynamic (Johnston et al., 1998). Soluble proteins found in aggresomes, including the aberrant proteins causing the formation of aggresomes, retain their mobility. Nuclear aggresomes are more dynamic than cytosolic aggresomes because cytosolic aggresomes contain more insoluble protein. This difference in solubility is likely due to fundamental structural differences between nuclear and cytosolic aggresomes. Both nuclear and cytosolic aggresomes appear to form from the coalescence of smaller aggregates that are moved along the microtubule tracks (Fu et al., 2005).
1.2.2 Chimeric Protein Markers

Chimeric proteins are protein constructs created by fusing different protein-coding regions together via subcloning (see Methods). Chimeric protein models are essential for fluorescently tagging wild-type TR (WT-TR), rc6-TR, v-ErbA, and two aggresomal markers: GFP-250 and GFP-170. The use of fluorescent tags allows for the visualization of protein location and distribution within a cell. A clear advantage of this method over comparable techniques, like immunofluorescence, is that the chimeric models used can be studied in live cells in real time.

GFP-250 is a bona fide, synthetic cytosolic aggresome marker (Garcia-Mata et al., 1999) used in this thesis research. It is a chimeric protein consisting of the entire green fluorescent protein (GFP) sequence fused at the COOH terminal to a 250 amino acid fragment of p115. Full length p115 is a cytosolic membrane transport protein which shuttles cargo between the ER and the Golgi body. Also used in this thesis research, GFP-170 is a chimera of Golgi Complex Protein 170 (GCP170) segment 566-1375 fused to GFP (Fu et al., 2005). GFP-170 localizes to both the nucleus and the cytoplasm but localizes to the nucleus to a greater extent than GFP-250, thus it was chosen as an indicator for nuclear aggresomes. Nuclear aggresomes are distinguishable because they overlap with DAPI, a fluorescent stain which binds to DNA.

1.3 Nucleocytoplasmic Transport

Eukaryotic cells have membrane bound organelles which allow for the compartmentalization and regulation of molecular processes within a cell. A prominent organelle is the nucleus which houses DNA. The membrane between the nucleoplasm and the cytoplasm is known as the nuclear envelope. The nuclear envelope is a highly selective, double membrane perforated with nuclear pore complexes (NPCs), which serve a distinct role in regulating what traffics into and
out of the nucleus. All nuclear proteins and all RNA must travel through the NPCs of the nuclear envelope; therefore, the nucleocytoplasmic transport pathway is consequential for all eukaryotic cells (Mor et al., 2014).

Imbalanced nucleocytoplasmic shuttling is correlated with tumorigenesis (Mor et al., 2014). For example, increased expression of exportins (see Section 1.3.3), like CRM1, can increase the export of tumor suppressors therefore inhibiting the efficacy of tumor suppressors and contributing to tumor formation. This overexpression of CRM1 has been found in gliomas, osteosarcomas, and leukemias. Changes in nuclear localization/export signals (see Section 1.3.1) can also have tumorigenic effects by altering the trafficking of vital proteins that alter gene expression, tumor suppression, and apoptosis.

1.3.1 Nuclear Localization Signals and Nuclear Export Signals

Nuclear localization signals (NLSs) are amino acid sequences which designate proteins for transport into the nucleus (Lange, 2006). The most well understood NLS, the classical NLS, consists of either one sequence of basic amino acids or two separate sequences of basic amino acids. These two types of NLSs are referred to as monopartite and bipartite, respectively. Monopartite NLSs follow the general pattern \texttt{PKKKRRV} (functional basic amino acids underlined), as exemplified by the SV40 large T antigen NLS. Bipartite NLSs follow the general pattern \texttt{KRPAATKKAGQAKKKK}, as exemplified by nucleoplasmin. TR\textsubscript{r} has two classical NLSs. NLS-1, in the hinge domain, is a bipartite NLS with the sequence: \texttt{KRVAKRKLIEQNRERRRK}\textsuperscript{147} (Mavinakere et al., 2012). NLS-2, in the A/B Domain, is a monopartite NLS with the sequence: \texttt{PDGKRKRK}\textsuperscript{29} (Mavinakere et al., 2012). TR\textsubscript{b} has a similar NLS-1 with the sequence: \texttt{KRLAKRKLIEENRERRRK}\textsuperscript{147} (Mavinakere et al., 2012). However, TR\textsubscript{b} does not have an NLS-2 equivalent.
Nuclear export signals (NESs) are amino acid sequences which designate proteins for transport out of the nucleus (Kutay and Guttinger, 2005). NESs are not as well characterized as NLSs, but classical NESs that have been described are sequences of up to 20 amino acids that are rich in leucine (L), valine (V), phenylalanine (F), isoleucine (I), and methionine (M) (Mavinakere et al., 2012; Kutay and Guttinger, 2005). TRα1 has at least three classical NESs, all found in the LBD. NES-H3/H6 form two separate NESs on two separate helices, but are joined by a Proline-Methionine sequence (underlined in sequence). The sequence for NES-H3/H6 is:

\[209^{\text{KVDLEAFSEFTKIITPAITRVVDFAKKLPMFSELPEDQIIL}}265\]

(Mavinakere et al., 2012). The third NES, NES-H12, is found on helix 12 and has the sequence:

\[390^{\text{VECPTELFPPLFLEV}}407\]

(Mavinakere et al., 2012). Additionally, TRα1 has an undefined NES that mediates export via a CRM1/calreticulin-mediated nuclear export pathway (Grespin et al., 2008).

**1.3.2 Nuclear Pore Complexes**

The nuclear envelope consists of an outer membrane and inner membrane which are connected by nuclear pore complexes (NPCs) that span the otherwise selectively permeable lipid bilayer (Kabachinski and Schwartz, 2015). NPCs are ~125 MDa and are composed of ~500 protein molecules which can be classified into ~30 different types of proteins that are collectively called nucleoporins (Nups). NPCs have an eightfold radial symmetry as well as a twofold symmetry across the nuclear envelope (Hoelz et al., 2011). NPCs consist of three rings: one cytoplasmic, one nuclear, and a core ring that lies in the intermembrane space. Cytoplasmic filaments emanate from the core into the cytoplasm while a basket-like structure protrudes from the core into the nucleoplasm. Both the cytoplasmic filaments and the nuclear basket provide sites for cargo to bind.
Despite a large size, the NPC is highly selective due to the FG-barrier (Kabachinski and Schwartz, 2015). The FG-barrier is composed of Nups in the core ring that have phenylalanine-glycine (FG) repeats. FG repeats are hydrophobic, filament like structures. FG-Nups have anywhere between five and 50 FG repeats, while the NPC core has ~200 FG-Nups. There are several models for nuclear transport through the FG-barrier. The strongest, most recent evidence favors the “selective-phase model”: FG repeats interact cohesively to form a sieve-like structure with a ~40 kDa sized mesh (Hoelz et al., 2011). Molecules larger than 40 kDa cannot penetrate the FG barrier without special carrier proteins which form weak bonds with FG-repeats that allow the carrier and its cargo to penetrate the FG-barrier.

### 1.3.3 General Nuclear Import and Export Pathways of TRα1

Nucleocytoplasmic trafficking is a highly regulated process (Pemberton and Paschal, 2005). In most cases, both nuclear import and export require Ran, a small GTP-binding protein. RanGTP is primarily nuclear due to the cofactor RanGEF (Ran Guanine Nucleotide Exchange Factor) being located in the nucleus. RanGEF replaces the GDP bound to RanGDP for a GTP. RanGDP is primarily cytosolic due to the cofactor RanGAP (Ran GTP-ase Activating Protein) which fosters the hydrolysis of RanGTP to RanGDP according to the reaction: 

\[
GTP \rightarrow GDP + Pi
\]

The concentration gradients of RanGTP and RanGDP control the directionality of import and export (Stewart, 2007). Carrier:cargo complexes shuttle rapidly through the NPC and establish an equilibrium which behaves according to Le Chatlier’s Principle (Stewart, 2007). For this reason, the gradient of Ran, which disrupts this equilibrium, controls the directionality of import and export.

NLSs and NESs in cargo proteins are recognized by importins and exportins, respectively, which are members of the karyopherin protein family. The classical model of nuclear import is
well characterized and involves two karyopherins: Importin-α and Importin-β1. Importin-β1, of the karyopherin-β family, can bind classical NLSs directly or through the adapter protein, Importin-α (Stewart, 2007).

Importin-α is composed of 10 armadillo (ARM) repeats with a flexible Importin-β Binding (IBB) domain on the N-terminus (Goldfarb et al., 2004). ARM repeats 1-4 serve as the binding site for monopartite NLSs and as the major binding site for bipartite NLSs. ARM repeats 6-8 serve as the minor binding site for bipartite NLSs (Stewart, 2007). The IBB domain is composed of a series of basic residues which mimic a low affinity NLS and can bind to NLS binding sites on Importin-α. This grants the IBB domain a dual-role: not only does it bind Importin-β1, but when not bound to Importin-β1, IBB competes with cargo for NLS binding sites, thus acting in an auto-inhibitory fashion. Importin α:β complexes, which have higher affinity for cargo than unbound Importin-α, bind cargo at a much higher rate. The generally accepted sequence of events for classic nuclear import thus becomes: Importin-α binds Importin-β1, and this complex then binds cargo (Figure 2).

The carrier:cargo complex enters the NPC where Importin-β1 interacts with FG repeats to penetrate the FG barrier through weak hydrophobic interactions (Stewart, 2007). Inside the nucleus, RanGTP binds Importin-β1 (which is complexed with Importin-α and the cargo) causing a conformational change which forces the Importin-α:cargo complex to be released. The IBB domain is now free to compete with the NLS binding site of Importin-α thus causing the cargo to dissociate. Additionally, Nup2/Nup50 increase cargo dissociation rates by binding Importin-α and competing for the NLS binding site of Importin-α in a similar manner to IBB.

Inside the nucleus, unbound Importin-α binds to RanGTP. The RanGTP:Importin-α complex is then bound by the exportin CAS (Pemberton and Paschal, 2005) (Figure 2). CAS is a member
of the Importin-β superfamily and is composed of 19 HEAT repeats (Stewart, 2007). CAS is similar in structure to Importin-β and also has an IBB domain. The IBB domain of CAS binds to the NLS binding sites of Importin-α which renders Importin-α unable to interact with cargo once bound to CAS. Additionally, CAS has two Ran-binding sites which independently form weak bonds with RanGTP. When CAS binds Importin-α, however, CAS undergoes a conformational change that allows both Ran-binding sites to make contact with RanGTP and allows for the export of Importin-α, CAS, and RanGTP.

Unbound Importin-β1 in the nucleus also binds RanGTP. The RanGTP:Importin-β1 complex is sufficient for nuclear export without CAS and is thus exported. In the cytoplasm, RanGAP assists Ran in hydrolyzing GTP, which causes the aforementioned complexes to dissociate. RanGDP is transported into the nucleus via Nuclear Transport Factor 2. The nuclear import cycle is now complete. CAS is recycled to the nucleus by an unknown pathway thus completing the nuclear export cycle (Stewart, 2007). The importins responsible for TRα1 nuclear trafficking are Importin 7, Importin-β1, and the adapter Importin-α1 (Anyetei-Anum et al., 2018, Roggero et al. 2016).
The Chromosome Region Maintenance 1 (CRM1) pathway is the classic nuclear export pathway (Hutten and Kehlenbach, 2007) (Figure 3). CRM1 is a karyopherin of the Importin-β superfamily and is composed of 19 HEAT repeats. HEAT repeat 8 has been indicated as the site for RanGTP binding and is also likely responsible for cooperative NES cargo binding. HEAT repeat 10 is commonly accepted as the primary NES binding site.

CRM1 requires RanGTP in order to form a stable export complex with NES cargo; however, the affinity of CRM1:RanGTP for NESs is low and is considered the rate limiting step of nuclear export (Hutten and Kehlenbach, 2007). RanBP3 enhances export complex formation in two ways. First, RanBP3, binds both RanGEF and CRM1 which increases the amount of RanGTP in the vicinity of CRM1 thus increasing the probability of forming an export complex. Secondly,
RanBP3 acts as co-factor for CRM1:RanGTP complexes by stabilizing the NES binding region of CRM1 thereby increasing the NES cargo binding rate. The CRM1:RanGTP:Cargo complex then dissociates from RanBP3 and diffuses through the NPC. In an analogous manner to import, the Ran gradient controls the directionality of export. In the cytoplasm, RanGAP assists Ran in the hydrolysis of GTP. The hydrolysis of GTP to GDP causes the dissociation of the export complex. CRM1 is thought to be shuttled back into the nucleus through undescribed interactions with Nups on the cytoplasmic side of the NPC.

It should be noted that for CRM1-dependent export of TRα1, the Ca^{2+}-binding protein, calreticulin is required (Grepsin et al., 2008) (Figure 3). Calreticulin interacts directly with TRα1, mediating the interaction with CRM1. TRα1 is capable of nuclear export without CRM1, because of the multiple pathways available for nuclear export. The exportins responsible for TRα1 nuclear trafficking are CRM1/calreticulin, exportin 4, exportin 5, and exportin 7 (Subramanian et al., 2015).
1.4 Specific Aims of Thesis

The specific aims of this thesis research were to:

1. Characterize the phenotypes of rc6-TRα1 and tc15-TRα1 in HeLa cells and describe the possible pathways contributing to these phenotypes.

2. Place the phenotypes of rc6-TRα1 and tc15-TRα1 in context with nucleocytoplasmic trafficking, mislocalization, and oncogenesis.
2. Methods

2.1 Fluorescent Fusion Protein Constructs

Human wild-type TRα1 and mutant constructs rc6-TRα1 and tc15-TRα1 coding regions were synthesized by GeneArt Gene Synthesis (Life Technologies), and cloned into GFP or mCherry expression plasmids (from Clontech) by Vincent Roggero. GFP-v-ErbA, GFP-250, and GFP-170 plasmids were previously constructed or gifted as described in Bondzi et al. (2011).

2.2 Plasmid Preparation

All plasmids were transformed in *E. coli*. 1ng of plasmid DNA was added to competent C2987H (New England BioLabs) *E. coli* cells and the mixture was inverted 5 times. The mixture was placed on ice for 10 minutes, then heat shocked for 30 seconds in a 42°C water bath. The mixture was then placed on ice for 3 minutes. 200μl of room temperature SOC medium was pipetted into the mixture and then 100μl was spread onto a Kanamycin-agar plate. The plate was incubated for 24 hours at 37°C.

Starter cultures containing 3mL of LB media and 30μg/mL of kanamycin were inoculated with single colonies from the plate. The starter cultures were grown at 300 rpm, 37°C for 8 hours. 1mL of the starter culture was used to inoculate a culture of 50mL of LB media and 30μg/mL of kanamycin which was grown to saturation at 300 rpm, 37°C for 24 hours. Cultures were then centrifuged at 6000xg for 10 minutes at 4°C. Plasmid was purified from the pellet using the Zymo Research Zyppy™ Plasmid Midiprep Kit. The purified plasmid was quantified using a Nanodrop® 1000 spectrophotometer.
2.3 Tissue Culture

HeLa cells (ATCC® CCL2™) were grown in Gibco™ Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) at 37°C, 5% CO₂, and 98% humidity. When cells reached a confluence of ~90%, the cells were washed with 1x D-PBS and dissociated with 0.25% trypsin before being passaged to a new flask or seeded for transfection.

2.4 Seeding and Transfection

HeLa cells were seeded at a density of 2.5x10⁵ cells/well onto coverslips (Fisherfinest™) in a six-well plate overnight. 2μg of plasmid DNA was diluted into 250μl of Opti-Mem. This solution was left to incubate for 5 minutes. In a separate tube, 24μL of Lipofectamine 2000 was mixed with 1500μl of Opti-MEM solution and incubated for 5 minutes. 250μl of the Lipofectamine-Opti-MEM solution was then added to each well, for the equivalent of 4μg of Lipofectamine, 2μg of DNA, and 500μl of Opti-MEM per well. For co-transfections, the amount of DNA was 4μg total, 2μg per respective plasmid.

After 8 hours of incubation at 37°C, 5% CO₂, and 98% humidity, the media solution was aspirated off each well. Next, 2ml of MEM supplemented with 10% FBS was added to each well. Cells were then incubated in the same conditions previously listed for 18 hours before being fixed.

2.5 Fixation and Staining of Slides

The media was aspirated off wells in a six-well plate and cells were washed three times for 15 seconds using 2mL of 1x D-PBS. Cells were then fixed for 6.5 minutes in 3.7%
formaldehyde solution in 1x D-PBS. The formaldehyde solution was removed and the cells were washed three times for 5 minutes in 2 mL of 1x D-PBS solution. Fluoro-Gel II with DAPI (Electron Microscopy Sciences) was used to mount the inverted coverslips to glass slides.

2.6 Fluorescence Microscopy and Scoring Slides

Slides (blinded by another lab member) were scored on an inverted fluorescence microscope using Nikon® NIS-Elements microscope imaging software. Two identical Regions of Interest (ROIs) were drawn in order to measure the fluorescence intensity of two areas: one in the nucleus and one in the cytoplasm. Cells with aggregates were not excluded, instead, ROIs were positioned such that they provided fluorescent intensities representative of the whole cell. The nucleus was visualized using the DAPI channel and the cytoplasm was visualized using the GFP or mCherry channel depending on which fluorescent tag was used. The mean nuclear to cytoplasmic ratio was determined through analysis in Microsoft Excel.

Three replicates were completed with a total of 100 cells scored per slide. A Student’s T-Test was used to test for a significant difference between the means of two samples. A significance level of $p \leq 0.05$ was considered significant.

2.7 Confocal Microscopy

Confocal microscopy was used to image aggregates and aggresomes in three dimensions. Confocal microscopy also allows for imaging on a specific Z plane. All data and images were collected using Nikon Elements. The lasers were optimized for each cell to eliminate pixel oversaturation. Data were collected using the Galvano setting except in extreme circumstances where Resonant was used to prevent fluorescent tag decomposition. Galvano scanning uses a dual mirror system to scan images between 4 and 50 frames per second (FPS). Resonant
scanning uses a singular, oscillating mirror to scan images between 30 and 60 FPS. The slower frame rate of Galvano allows for higher laser dwelling on each picture and thus higher quality images. Occasionally, higher pixel dwell can cause fluorescent tags to photo-bleach thus Resonant was used to prevent photo-bleaching in extreme circumstances. Line averaging was used to increase pixel resolution. Additionally, the Nyquist feature was used to focus on specific cells. The Nyquist feature adjusts the sampling frequency such that it is twice the band width of the input signal. This allows the image generated to be a perfect representation of the sample. Channel lock was used for all data collection and imaging to eliminate any bleed-through from different channels.

2.8 Colocalization Analysis

Pearson’s Correlation Coefficient (PCC) was used to assess the relationship between fluorescent intensities of two fluorophores, also known as colocalization. The formula is as follows:

\[ r = \frac{\sum (R_i - R_{av}) \cdot (G_i - G_{av})}{\sqrt{\sum (R_i - R_{av})^2 \cdot \sum (G_i - G_{av})^2}} \]

The numerator is the sum of the products of the two intensities (red and green) and is maximized when the relative intensities coincide (high intensity red with high intensity green, low intensity red with low intensity green). A combination of high and low intensities of either fluorophore will yield a smaller sum. The denominator yields the maximum product of the sample thus limiting \( r \) to a number between -1 and 1. A standard threshold of significant correlation, \( r \geq 0.5 \), was used (Adler and Parmyrad, 2010). Oval Regions of Interest (ROI’s) were generated by hand.
around foci in merged GFP-mCherry images. The ROI’s were drawn so that some area around the foci was also captured. This was done in order for the PCC to be measured accurately.

Three replicates were completed with 30 cells per slide imaged. Each cell contained ~5 aggregates and/or one large aggresome that were scored. Images of slides were collected on a confocal microscope to ensure that all foci were on the same Z plane. Importantly, the channel lock was engaged so that any bleed-through between channels would not be captured. The lasers for each respective channel were optimized so that pixel oversaturation did not occur. These image planes were then combined and analyzed using Nikon Elements Colocalization Tool.

3. Results

3.1 rc6-TRα1 has a significantly greater cytoplasmic localization than wild-type TRα1

rc6-TRα1 is a dominant-negative, TR mutant that was originally isolated from patients with renal clear cell carcinoma (Rosen et al., 2011). rc6-TRα1 has three amino acid substitutions: I116N, A225T, and M388I (Figure 1). A225T is a mutation in NES-H3/H6 leading to the hypothesis that rc6 would have altered nucleocytoplasmic shuttling compared to wild-type TRα1. The prediction was that rc6 would show increased nuclear localization relative to wild-type TRα1. To test if the nuclear localization of rc6-TRα1 was significantly different from the wild-type TRα1, Hela cells were transfected with expression plasmids for GFP-rc6-TRα1 and mCherry-TRα1 (Figure 4).
Figure 4. (A) 100 cells were scored per viable slide for a total of 700 cells, over three replicates, per treatment group. Scale bar, 10m (B) Average nuclear to cytoplasmic ratio of rc6-TRα1 and TRα1. * indicates a p value < 0.05. p= 0.004, SEM= 0.18 and 1.09, respectively.
Contrary to the predication, rc6-TRα1 has a statistically significant cytosolic shift compared to wild-type TRα1 (Figure 4B), suggesting that rc6-TRα1 displays a phenotype characterized by mislocalization. Additionally, rc6 has a large amount of aggregates which form multiple different patterns (Figure 5). The cytosolic shift of rc6 and the substantial presence of aggregates phenotypically resembles the distribution pattern of the oncoprotein v-ErbA.

Figure 5. rc6-TRα1 cytosolic and nuclear aggregates and aggresomes. Images are representative of commonly observed phenotypes. Scale bar, 10μm
3.2 rc6-TRα1 colocalizes with v-ErbA

The phenotypic resemblance between rc6-TRα1 and v-ErbA led to the prediction that rc6-TRα1 may follow similar mechanisms and pathways within the cell to the known oncoprotein, v-ErbA. To test for colocalization, Hela cells were transfected with expression plasmids for mCherry-v-ErbA and GFP-rc6-TRα1. Thirty cells were scored per slide, two slides per replicate, for a total of three replicates. Each cell scored contained multiple aggregates and/or one fully formed aggresome. The minimum number of aggregates and aggresomes scored per replicate was 110, with one replicate yielding 140 aggregates and aggresomes scored.

rc6-TRα1 and v-ErbA colocalize in aggregates and aggresomes with a significant Pearson’s Correlation Coefficient (PCC) of 0.92. This relationship between rc6 and v-ErbA warranted further investigation of the nature of the aggregates forming in rc6-TRα1.
3.3 rc6-TRα1 colocalizes with aggresomal markers GFP-250 and GFP-170

To determine whether rc6-TRα1 was recruited to bona fide aggresomes, Hela cells were cotransfected with sets of two expression plasmids: mCherry-rc6-TRα1 and GFP-250 (Figure 7A), or mCherry-rc6-TRα1 and GFP-170 (Figure 7B). GFP-250 and GFP-170 are bona fide cytosolic and nuclear aggresome markers, respectively.
The high correlation between rc6-TRα1 and both aggresomal markers further highlights the similarity in phenotype between rc6-TRα1 and v-ErbA.

3.4 rc6-TRα1 single mutation phenotypes have wild-type N/C ratios

rc6-TRα1 contains three single amino acid substitutions, I116N in the DBD and A225T and M388T in the LBD. It was hypothesized that each point mutation would have an impact on TRα1 localization. Specifically, I116N was predicted to have a greater cytosolic shift, because
DNA binding may be impaired by the mutation leading to lower nuclear retention. A225T was predicted to have greater nuclear localization because the mutation is within NES-H3/H6 and is perhaps deleterious to proper NES function. M388I, which flanks NES-H12 was predicted to impede NES function and thus have greater nuclear localization.

Individual wells of Hela cells were transfected with expression plasmids for GFP-TRα1-I116N, GFP-TRα1-A225T, GFP-TRα1-M388I, GFP-rc6-TRα1, GFP- tc15-TRα1, and GFP-TRα1 (Figure 8).
Interestingly, none of the point mutations had altered nucleocytoplasmic localization. tc15-TRα1, a mutant found in thyroid cancer (Puzianowska-Kuznicka et al., 2002), with mutations

**Figure 8.** Nucleocytoplasmic distribution of TRα1 mutants: TRα1-A225T, TRα1-M388I, TRα1-I116N, tc15-TRα1. (A) All slides were blinded and 100 cells were scored per slide for three replicates. The images for I116N and A225T are representative of ~60% of cells. Scale bar, 10 μm (B) Matrix of Student’s T-Tests performed with each average N/C ratio.
S183N, H184Q, and R228H also had normal nucleocytoplasmic localization (Figure 8B, Figure 9). The only significant difference in localization occurred between rc6-TRα1 and TRα1. Of great interest was that I116N and A225T had high numbers of aggregates compared to wild-type TRα1, so much so that they were indistinguishable from rc6-TRα1.

![Figure 9](image)

**Figure 9.** Average nuclear to cytoplasmic ratio of tc15-TRα1 and TRα1. 100 cells were scored per replicate for 3 replicates for a total of 300 cells per protein. SEM= 0.21 and 0.81 respectively, p>0.05.

4. Discussion

4.1 Summary

A major finding of this thesis research is that rc6-TRα1, a mutant form of TR associated with renal cell carcinoma, has a higher cytosolic localization than wild-type TRα1 when expressed in HeLa cells. However, all three single point mutations (I116N, A225T, M388I) must be present in
order for this phenotype to occur. Additionally, rc6-TRα1 forms aggregates and aggresomes that colocalize with the bona fide aggresome markers GFP-250 and GFP-170. When cotransfected, rc6-TRα1 also colocalizes with the oncoprotein v-ErbA. Both I116N and A225T amino acid substitutions are sufficient on their own to form aggregates and aggresome-like structures, although neither I116N nor A225T had altered nucleocytoplasmic shuttling.

In contrast, a mutant TR associated with thyroid cancer, tc15-TRα1 (S183N, H184Q, R228H), showed no significant change in localization compared to wild-type TRα1. tc15-TRα1 also lacks the aggregate formation and aggresome recruitment that characterizes the phenotype of rc6-TRα1.

These results provide insight into the mechanisms by which rc6-TRα1 and tc15-TRα1 may contribute to the pathogenesis of renal clear cell carcinoma and thyroid papillary carcinoma, the cancers from which these respective mutants originate. Characterizing the cellular effects of rc6-TRα1 and tc15-TRα1 and the pathways by which these effects may occur is an important step toward understanding and treating these cancers.

4.2 Aggresomes: A Pathway to Oncogenesis?

Aggresomes are dynamic structures in which aberrant proteins, at least in some cases, still maintain mobility and thus can shuttle to and from the aggresomes (Bondzi et al., 2011). Shuttling to and from the aggresome adds an alternate pathway for rc6-TRα1 to travel, thus decreasing the amount available to shuttle into and out of the nucleus. This holds true for both nuclear and cytosolic aggresomes. The dynamic trafficking of rc6-TRα1 between the nucleus, cytoplasm, and aggregates/aggresomes may have profound effects on gene regulation. rc6-TRα1 is a dominant negative inhibitor of wild-type TRα1 and therefore may be recruiting wild-type
TRα1 into the aggresome-shuttling pathway, thereby decreasing the amount of nuclear wild-type TRα1. rc6-TRα1 also has an increased repertoire of gene targets, compared to the wild-type, that are upregulated and downregulated (Rosen et al., 2011). This means that rc6-TRα1, when present in the nucleus, is abnormally altering gene expression regardless of the dominant negative effects it exhibits on wild-type TRα1. The culmination of these effect likely causes significant genetic misregulation, possibly leading to oncogenesis. v-ErbA follows a similar pathway for its oncogenic effect (Bondzi et al., 2011). As shown, rc6-TRα1 has a high colocalization with v-ErbA and may follow a similar pathway that contributes to oncogenesis.

Aggregates are generally formed from exposed hydrophobic amino acids. None of the three mutations of rc6-TRα1 (I116N, A225T, M388I) involve a direct substitution to a more hydrophobic amino acid. For I116N, the amide group on the R chain of asparagine is capable of accepting and donating hydrogen bonds, a feature that isoleucine lacks. This may indicate that I116N, which is capable of inducing aggregates, is creating a conformational change in TRα1 that exposes a hydrophobic region. For A225T, the hydroxyl group of the R chain of threonine is capable of donating a hydrogen bond, a characteristic not shared by alanine. A225T, which is also capable of forming aggregates, may also induce a conformational change in TRα1 by a similar mechanism to I116N. M388I, however, does not appear to be capable of consistently forming aggregates. Isoleucine carries no charge and is incapable of forming hydrogen bonds. There could be steric changes that induce conformational change, however, it appears unlikely that M388I produces an appreciable change in structure on its own.

The cumulative effects of the three mutations culminate in aggregate formation and recruitment to the aggresome, as well as increased cytosolic localization. Whether the increase in
cytosolic localization is solely the product of recruitment to the aggresome requires further study, but it seems likely that aggresome presence contributes significantly to this phenotype. The phenotype of the point mutations I116N and A225T can perhaps be explained by the proportionality of cells with aggregates and aggresomes versus those without. Additionally, the point mutants had high biological variance. After removing outliers from the point mutation experiment data sets, the difference between the means of I116N and wild-type TRα1 became significant at a 95% confidence interval (see Appendix).

4.3 Future Directions

The high biological variance observed in the point mutant experiments with I116N, A225T, and M388I requires a larger sample size in order to draw more robust conclusions from the N/C measurements. More replicates should be completed to ensure a normalized distribution of data. Another important question is how the presence of biologically relevant levels of T3 affect the
phenotype of rc6-TRα1 and tc15-TRα1. N/C comparisons of mutant TRα1 in the presence and absence of T3 could further define the phenotype of rc6-TRα1 and tc15-TRα1. Further experiments should quantify the colocalization, using PCC, of wild-type TRα1 with rc6-TRα1 and tc15-TRα1, respectively. Comparing the N/C ratio of the wild-type when cotransfected with a mutant (rc6-TRα1 or tc15-TRα1) to the non-cotransfected wild-type could provide a semi-quantitative description of the dominant-negative activity of the mutants with regards to changes in intracellular localization patterns.

5. Appendix

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6. References


