Receptor Gone Rogue: Investigating Localization of a Cancerous Thyroid Hormone Receptor

Michelle Barbeau

College of William and Mary

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Part of the Cancer Biology Commons, and the Cell Biology Commons

Recommended Citation


https://scholarworks.wm.edu/honorstheses/1024
**Abstract**

Thyroid hormone receptors play a crucial role in regulating differentiation, growth and development in response to thyroid hormone, and mutations in these receptors can have severe medical consequences ranging from endocrine dysfunction to cancer. Tumors of patients with hepatocellular carcinoma (HCC) display a high incidence of mutant thyroid hormone receptors (TRs), and one such mutant is TRα1 (K74E, A264V). The binding partners and gene targets of this mutant have been characterized, but the role of intracellular localization in the pathogenesis of TRα1 (K74E, A264V) has not yet been determined. Here, it was observed that the mutant receptor has a tendency to aggregate when transfected into HeLa (human) cells. Fluorescence microscopy was used to determine the extent of this aggregation. The data showed that the TR mutant displays a significantly higher frequency of nuclear and cytosolic aggregates. Furthermore, it induces a significantly higher amount of nuclear and cytosolic aggregates in the wild type TR when coexpressed in the cell, and this effect increases with increasing amounts of transfected mutant-containing plasmid. These results highlight a potential dominant-negative of TRα1 (K74E, A264V) effect as it pertains to localization and offer a new layer of understanding to the altered activity of this mutant TR during the development of cancer.
**Table of Contents**

**Introduction**.......................................................................................................................... 1  
Thyroid Hormone......................................................................................................................... 1  
Thyroid Hormone Receptors........................................................................................................ 2  
  Structure .................................................................................................................................... 2  
  Mechanism of Action ................................................................................................................ 4  
Thyroid Hormone Receptors and Cancer .................................................................................... 6  
  Oncogenesis ............................................................................................................................. 6  
  Hepatocellular Carcinoma and TRα1 (K74E, A264V) ............................................................. 7  
  Localization .............................................................................................................................. 8  
Research Aims ............................................................................................................................ 8  

**Materials and Methods** ........................................................................................................ 8  
Plasmids ....................................................................................................................................... 8  
Cell Culture and Transfections .................................................................................................. 9  
Fixation and Staining .................................................................................................................. 9  
Phenotypic Categorization and Statistical Analysis ..................................................................... 9  

**Results** ................................................................................................................................... 10  
   TRα1 (K74E, A264V) forms significantly more aggregates than its wild type counterpart ......... 10  
   Wild type TRα1 displays nuclear and cytosolic aggregates when coexpressed with TRα1 (K74E, A264V) ........................................................................................................................................ 13  
   Increased expression of TRα1 (K74E, A264V) increases wild type aggregation ..................... 17  

**Discussion** ............................................................................................................................... 22  
   TRα1 (K74E, A264V) displays nuclear and cytosolic aggregation ............................................... 22  
   TRα1 (K74E, A264V) induces aggregate formation in TRα1 ......................................................... 23  

**Future Directions** .................................................................................................................. 25  

**References** .............................................................................................................................. 27  

**Appendix A** .............................................................................................................................. 30  
**Appendix B** .............................................................................................................................. 30
List of Figures

Figure 1: Structure of thyroid hormone ................................................................. 2
Figure 2: Major TR splice variants .................................................................. 3
Figure 3: General layout of the TR domains .................................................... 4
Figure 4: TR mechanism of action .................................................................. 5
Figure 5: TRα1 (K74E, A264V) ................................................................. 7
Figure 6: Categories of phenotypes for GFP-TRα1 (K74E, A264V) ............ 11
Figure 7: Quantitative comparison of wild type TRα1 and mutant TRα1 (K74E, A264V) cellular distributions .......................................................... 12
Figure 8: Phenotypic categories used for quantifying cotransfection aggregation .............. 15
Figure 9: Cotransfection trials .................................................................. 16
Figure 10: Control analysis of GFP-TRα1 (K74E, A264V) alone and with mCherry-TRα1 .......... 17
Figure 11: Perinuclear aggregates ............................................................... 19
Figure 12: Analysis of the phenotypes of wild type TR in cells transfected with an increase of TRα1 (K74E, A264V) plasmid .................................................... 20
Figure 13: Analysis GFP-TRα1 (K74E, A264V) as a control to the analysis of wild type distribution in response to an increase in mutant plasmid ............................................ 20
Acknowledgements

A huge thank you goes out to my incredible research advisor, Dr. Allison, for guiding me and giving me the opportunity to learn, to grow, and to pursue this thesis. Thanks are also owed to my committee members, Dr. Dalgleish, Dr. Saha, and Dr. Meldrum, for all of their help and support. I would also like to thank lab manager Vinny Roggero for his endless patience in the lab, showing me the ropes and helping me through my various technical issues. I am also grateful for the Allison Lab members for being a constant source of collaboration and commiseration.

A special thank you goes out to the Charles Center and all those who funded my research project, enabling me to focus on my research full-time over the summer. I would not be where I am today without the love and support of my family, especially my dad who is my biggest fan and cheerleader.
Introduction
Big Picture

The goal of this honors thesis is to look at a cancerous thyroid hormone receptor from a different angle, specifically where it is located within the cell. The localization of a protein is instrumental in its ability to carry out its physiological function, and altered localization can lead to pathology (Hung and Link, 2011). To understand the significance of this research, it is first important to understand the details on thyroid hormone receptor’s function, its mechanism, its link to cancer and the mutant studied in this thesis, and how localization can play into the cancerous phenotype.

Thyroid Hormone

The story of thyroid hormone receptors begins with the genesis of thyroid hormone, regulated by the hypothalamic-pituitary axis in the brain (Mullur et al., 2014). The initial signal to increase thyroid hormone production comes from the secretion of thyrotropin-releasing hormone (TRH) by the hypothalamus. TRH acts on the pituitary gland to induce secretion of thyroid stimulating hormone (TSH). TSH then acts on the thyroid gland to stimulate the production and release of thyroid hormone in the forms of T₃ and T₄, with the three and the four referring to the number of iodine groups the hormone contains (Figure 1). The thyroid produces the longer-lived T₄ in higher quantities than T₃, though T₃ is considered to be the more active form of thyroid hormone. T₄ is converted to T₃ locally at or in the target tissue through deiodinases D1 and D2 or to the more inactive reverse T₃ (rT₃) (Dentice et al., 2013). When the thyroid hormone enters a target cell, through specific transporters such as monocarboxylate transporter 8 (MCT8), organic anion-transporting polypeptide 1C1 (OATP1C1), or other related transporters, it can interact with its receptor (Visser et al., 2011).
Thyroid Hormone Receptors

Structure

Thyroid hormone receptors (TRs) are nuclear receptors which act as transcription factors, modulating gene expression based on ligand binding. Humans have two TR genes, THRA and THRB, which are differentially expressed based on tissue type and stage in development (Pascual and Aranda, 2013). Each gene encodes multiple distinct receptors through differential RNA splicing (Figure 2). THRA encodes one T₃-binding splice variant, TRα1, and two non-T₃-binding splice variants, TRα2 and TRα3, along with several other truncated forms. THRB codes for three major T₃-binding splice products, TRβ1, TRβ2, and TRβ3. All of these variations on the TR theme are part of the reason why thyroid hormone can have selective actions on certain cell types. The basic structure of TR and of nuclear receptors in general involves four broad domains (Figure 3). The first domain is an amino-terminal “A/B” domain which recruits coregulatory proteins (Rosen and Privalsky, 2011). This domain also contains a nuclear localization signal (NLS), named NLS-2, in TRα1, which allows for the import of the TR into the nucleus through the nuclear pore complex. (Mavinakere et al., 2012). The second domain is the “C” domain, or
the DNA-binding domain (DBD) where the receptor can interact with specific nucleotide sequences in the genome. The “D” domain is the third domain, and it is also known as the hinge region, acting as a flexible link that joins the “C” and the “E/F” domains. In TRα1 and TRβ1, the hinge region also contains an NLS known as NLS-1 (Mavinakere et al. 2012; Lee and Mahdavi, 1993). The last domain, a carboxyl-terminal ligand-binding domain (LBD) also known as the “E/F” domain, is where ligands such as T₃ can bind and induce conformational changes in the rest of the protein to alter the receptor’s activity. Additionally, this site forms a receptor dimerization surface, acts as a major site for coregulatory interaction, and contains multiple nuclear export signals (NESs), (NES-H12), (NES-H3), and (NES-H6), named for the helical structures on which they reside (Mavinakere et al., 2012). The NESs allow for TR shuttling out of the nucleus.

Figure 2: Major TR splice variants (modified from Ortiga-Carvalho et al. 2014).
Mechanism of Action

TRs act as dimers, either binding to different nuclear receptors such as retinoid acid receptor (RXR) in a heterodimer or to other TRs in a homodimer (Brent, 2012) (Figure 4). The dimer binds to DNA sequences known as T₃ response elements (TREs) and regulates the expression of associated genes in response to thyroid hormone by recruiting coactivators when up-regulating gene expression or corepressors when down-regulating gene expression. TRs not bound to T₃ have a repressive effect on genes positively regulated by T₃ and an activating effect on genes normally repressed by T₃. TRs accumulate in the nucleus, though they rapidly shuttle in and out via importins and exportins (Bunn et al., 2001; Subramanian et al., 2015; Roggero et al., 2016).
Figure 4: TR mechanism of action (modified from Brent, 2012).
Thyroid Hormone Receptors and Cancer

Oncogenesis

The link between thyroid hormone receptors and cancer was first established in chickens infected with avian erythroblastoma virus (AEV). One of the viral genes that contributed to oncogenic (cancerous) transformation was a highly mutated TRα1 ortholog, v-ErbA (Thormeyer and Baniaahmad, 1999). The key mutations involved in differentiating oncogenic TRs from normally functioning ones are found in two domains, the DNA-binding domain and the ligand binding domain. When there is a significant mutation in the DNA binding domain, not only will the TRs bind to a subset of their usual TREs, but they also acquire, and therefore misregulate, new gene targets which could promote neoplasia (Rosen and Privalsky, 2011). TRs have a role in tumor suppression, and losing that role also promotes tumorigenesis (Wu et al., 2013; Zhu et al., 2010).

Significant mutations in the ligand-binding domain could either prevent or hamper T3 binding to block the protein’s ability to “sense” the levels of ligand within the cell. In these cases, not only can the TR not activate genes positively regulated by T3 and repress genes negatively regulated by T3, but it also continuously silences or activates genes, which should be regulated this way in the absence of T3, even when thyroid hormone is present in abundance. Furthermore, loss of function can occur when there is a normally functioning wild-type TR counterpart. The mutant TR will display dominant-negative activity, competing with the wild type for TRE binding and preventing the normal regulatory activity of the wild type TR (Kim and Cheng, 2013). This two-pronged attack to normal cellular functioning is a trademark of oncogenic TRs.
**Hepatocellular Carcinoma and TRα1 (K74E, A264V)**

Liver cancer such as hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality (El-Serag et al., 2007). HCC is most commonly linked with cirrhosis, and the major risk factor involved is hepatitis B and C viral infection. Interestingly, TRs with point mutations were detected with high frequency in human HCC tumors (Lin et al., 1999). TRα1 (K74E, A264V) is one such TR implicated in HCC is the focus of this research, displaying mutations in the typical areas leading to oncogenicity (Figure 5). The lysine at position 74 is a conserved residue in the DNA-binding domain which acts as an allosteric sensor, regulating transcriptional activity in response to DNA binding (Chan and Privalsky, 2010). In this mutant, the positively-charged lysine is exchanged for the negatively-charged glutamate, effecting a substantial conformational shift in a crucial area of the protein. This causes the mutant to display an altered target gene repertoire leading to the misregulation of tumor suppressor genes, oncogenes, and genes involved with cell migration and metastasis (Chan and Privalsky, 2009). Additionally, the alanine at position 264 in the ligand-binding domain is mutated to the bulkier valine, resulting in a delayed corepressor release and reduced sensitivity to T3 (Rosen and Privalsky, 2011).

![Figure 5: TRα1 (K74E, A264V)](image)
Localization

This thesis starts at the intersection of cancer and localization. The second mutation in TRα1 (K74E, A264V) is in NES H3/H6 (spanning amino acid residues 209-265), making it of interest for localization studies (Mavinakere et al., 2012). Previous research has shown that the oncoprotein v-ErbA is mislocalized; much of v-ErbA remains cytoplasmic due in part to an acquired viral NES (DeLong, et al., 2004). Furthermore, it displays a dominant-negative activity in that it binds to TRα and sequesters a significant fraction in the cytoplasm thus preventing wild type TRα from binding TRE unlike conventional TRE competition (Bonamy et al., 2005). Consequently, localization can play a large role in the oncogenic actions of mutant TRs.

Research Aims

This thesis explored whether TRα1 (K74E, A264V) is mislocalized within the cell relative to wild type TRα1, and if so, whether the mislocalization has a dominant-negative effect on wild type TRα1.

Materials and Methods

Plasmids

GFP-TRα1 and the mCherry-TRα1 expression plasmids were previously described (Bunn et al., 2001; Bondzi et al., 2011). The TRα1 (K74E, A264V) parent plasmid was obtained through GeneArt. GFP-TRα1 (K74E, A264V) was prepared by subcloning the TRα1 (K74E, A264V) cDNA into a GFP vector, and mCherry-TRα1(K74E, A264V) was prepared by subcloning the
TRα1(K74E, A264V) coding region from GFP-TRα1(K74E, A264V) into mCherry C1(Clontech).

**Cell Culture and Transfections**

HeLa cells (human cervical epithelioid carcinoma) were grown and maintained at 37°C and 5% CO₂ in Minimum Essential Medium (Gibco), supplemented with 10% fetal bovine serum (Invitrogen). For each transfection, 2.5 x 10⁵ HeLa cells were seeded into each well of a 6-well plate with glass coverslips (Fisher) and incubated at 37°C for approximately 24 hrs. Various amounts of plasmid expression vectors were introduced using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations (Appendix A). Cells were fixed 24 - 28 hrs post-transfection and analyzed by fluorescence microscopy.

**Fixation and Staining**

Cells were fixed approximately 24-28 hrs post-transfection. After aspirating off the media, each well was washed with 2mL 1X D-PBS solution in 3 rounds of 15-second washes (Appendix B). The cells were then fixed with a solution of 2mL 3.7% formaldehyde for 10 minutes. This was followed by 3 washes of 2mL 1X D-PBS for 2 minutes each. Coverslips were then mounted onto microscope slides in Fluoro-Gel II (Electron Microscopy Sciences) containing the DNA stain DAPI (4, 6-diamino-2-phenylindole).

**Phenotypic Categorization and Statistical Analysis.**

Images were collected from an inverted Nikon ECLIPSE TE 2000-E fluorescent microscope (Sigma, Melville, NY) using a 40X objective lens and acquired using NIS-Elements AR software.
The filters used to visualize localization patterns were the UV-2E/C filter block for the DAPI, the FITC (blue light) B-2E/C filter block for GFP, and the TRITC (green light) T-2E/C filter block for the mCherry.

Before the slides were analyzed, they were blinded by another lab member to control for bias. At least four replicate trials were performed, with a minimum of two slides per replicate and 200 cells categorized per slide averaged out for each replicate. Results were tested for statistical significance using a Student’s T-test, and a p-value of less than 0.05 was considered to be significant.

**Results**

**TRα1 (K74E, A264V) forms significantly more aggregates than its wild type counterpart**

Previous research has indicated that an altered localization (including association with aggresomes) could contribute to the oncogenic properties of v-ErbA (Bondzi et al., 2011), therefore a similar approach was taken with the mutant TRα1 (K74E, A264V). In order to visualize this protein, two GFP expression vectors were used to fluorescently tag the mutant and the wild type TRα1. HeLa cells were transfected with either 2μg of the wild type or the mutant plasmid, fixed, stained, and then viewed under a fluorescent microscope. After preliminary analysis of the mutant TR cellular distribution, a high incidence of protein aggregation was noted. This is in direct contrast with the fairly consistently even distribution of the wild-type TR. Therefore, in order to quantify the significance of this deviation from normal distribution, a count of the varying phenotypes was conducted. Three main phenotypes were defined as categories for scoring (Figure 6). “Nuclear aggregates” describes cells which have aggregates
clearly in the nucleus either by fluorescent delineation or by overlap with the DNA stain DAPI. “Cytosolic aggregates” encompasses a whole cell distribution of aggregates or covering a range greater than that demarcated by the DAPI stain. Finally, “even” refers to a lack of significant aggregates in that cell. After five trials of categorizing cells (n=200), the wild type cells displayed an average of 196.7 out of 200 even-distribution cells, 0.1 cells with nuclear aggregates, and 2.9 cells with cytosolic aggregates (Figure 7). The mutant, on the other hand, showed an average of 154 cells out of 200 with an even distribution, 27.2 cells with nuclear aggregates, and 18.8 cells with cytosolic aggregates.

![Figure 6](image_url)

**Figure 6:** Categories of phenotypes for GFP-TRα1 (K74E, A264V) (green). The nucleus is shown in blue (DAPI stain). A) Even distribution B) Nuclear aggregates C) Cytosolic aggregates.
Figure 7: Quantitative comparison of wild type TRα1 and mutant TRα1 (K74E, A264V) cellular distributions. Bars indicate standard error of the mean (SEM).

The differences between the wild type and the mutant were analyzed using a Student’s T-test, generating p-values of 3.07e-6, 0.004, and 0.002 for the categories of even distribution, nuclear aggregates, and cytosolic aggregates, respectively.

Aggregation indicates a high level of misfolded proteins within the cell. Aggregates are commonly nonhomogeneous and may contain chaperone molecules, proteasome subunits, glycosaminoglycans (GAGs), and proteoglycans other than the specific insoluble proteins (Bartolini and Andrisano, 2010). The recruited molecules appear to promote aggregate formation and reduce its clearance. Aggregates can pose problems for cells, mediating toxicity and impeding normal cellular functions. Nuclear aggregates were rarely seen in the cells transfected with wild type TRα1, and this may be a method of regulating nuclear retention of TRα1 (K74E, A264V). It could be a cellular way to prevent DNA binding while chaperones and proteasomes
work to bring down the level of misfolded protein in the nucleus. Alternatively aggregates can act as rapidly reforming and restructuring entities, so it is possible mutant TRs in the nucleus could switch between DNA-binding to fusion with aggregates (Fu et al., 2005). Cytosolic aggregates could also sequester the mutant TR in the cytoplasm, preventing transcriptional regulation.

As a qualitative observation, the cells with GFP- TRα1 (K74E, A264V) distributing evenly throughout the cell appeared to have a reduced fluorescence in contrast to the bright fluorescence seen in cells displaying aggregates. This might indicate a threshold level of expression before significant aggregate formation. On the other hand, this could simply be the result of a concentration of otherwise diffuse fluorescent molecules. The punctate nature of the aggregates makes quantification of relative fluorescent intensity more of a challenge.

**Wild type TRα1 displays nuclear and cytosolic aggregates when coexpressed with TRα1 (K74E, A264V)**

After demonstrating that TRα1 (K74E, A264V) forms aggregates, the next step was to ascertain whether the mutant TR aggregates could interfere with the localization of wild type TRα1. In order to do this, mCherry-TRα1 plasmids were used to distinguish the wild type TR when it was co-transfected with the GFP-TRα1 (K74E, A264V) plasmid. Cells were transfected with 2μg of wild type DNA alone, 2μg of mutant DNA alone, or 1μg of wild type DNA and 1μg of mutant DNA simultaneously. The same categories were used as before, and it was determined that the wild type TR exhibited altered localization when coexpressed with the mutant (Figure 8). When
expressed alone in the cell, mCherry-TRα1 had an average of 196.6 out of 200 cells with an even distribution, 0.9 cells with nuclear aggregates, and 2.5 cells with cytosolic aggregates (Figure 9). When coexpressed with GFP- TRα1 (K74E, A264V), mCherry-TRα1 had an average of 181.9 cells with an even distribution, 12.1 cells with nuclear aggregates, and 5.4 cells with cytosolic aggregates. Differences between the two conditions were once again analyzed using a Student’s t-test, yielding p-values of 7.78e-05, 8.92e-05, and 0.09 for the categories of even distribution, nuclear aggregates, and cytosolic aggregates, respectively. This demonstrates that the number of cells with evenly distributed wild type TRs and cells with wild type TRs aggregating in the nucleus were significantly different between the control and the co-transfected groups.
Figure 8: Phenotypic categories used for quantifying cotransfection aggregation.
To ensure that the aggregation of mCherry-TRα1 was not due to aberrant mutant activity within the cell, the differences between GFP-TRα1 (K74E, A264V) expressed alone and with mCherry-TRα1 were analyzed as a control (Figure 10). When only GFP-TRα1 (K74E, A264V) was transfected, an average of 158.9, 16.6, and 24 out of 200 cells displayed an even distribution, nuclear aggregates, and cytosolic aggregates respectively. When GFP-TRα1 (K74E, A264V) was co-transfected with mCherry-TRα1, an average of 176.9, 9.5, and 13.6 out of 200 cells presented an even distribution, nuclear aggregates, and cytosolic aggregates respectively. Using Student’s t-test, p-values of 0.04 for the even distribution, 0.19 for nuclear aggregates, and 0.05 for cytosolic aggregates were obtained. Transfection of mutant TR resulted in significantly more cells with an even distribution and significantly less cells with cytosolic aggregates when co-transfected with the wild type TR (using 1μg of mutant plasmid DNA), in contrast with the
mutant TR transfected alone using 2μg of plasmid DNA. There was no significant difference between the two conditions for nuclear aggregates. As there was no increase in mutant aggregates in the co-transfected cells, it is unlikely to have affected the significance of the previous analysis.

These results suggest that TRα1 (K74E, A264V) could be sequestering wild type TRα1 in these aggregates, whether in the cytosol or in the nucleus. This would add an additional means to dominant negative action by the mutant TR, comparable to v-ErbA (Bondzi et al., 2011).

**Increased expression of TRα1 (K74E, A264V) increases wild type aggregation**

The next step was to determine whether the dominant-negative action of TRα1 (K74E, A264V) would increase upon increased expression. The same expression vectors were used, but with 3μg
of wild type DNA, 3μg or mutant DNA, and for the cotransfection, 1μg of wild type DNA per 2μg of mutant DNA. Preliminary analysis determined that a new phenotypic category needed to be added for scoring. In addition to the previous aggregate distribution, a significant number of cells displayed “perinuclear” aggregates where the aggregates are neither strictly within the bounds of the nucleus nor are they spread throughout the cell (Figure 11). Instead, these aggregates encompass the nuclear region delineated via fluorescence or DAPI along with the cytoplasm directly near and around the nucleus. Cells were quantified using these four categories (Figure 12). Wild type TR expressed alone presented an average of 197.5, 0.9, 0.4, and 1.2 out of 200 cells with an even distribution, nuclear aggregates, perinuclear aggregates, and cytosolic aggregates respectively. Wild type TR expressed with twice the amount of mutant TR displayed an average of 154.4, 19.4, 15.9, and 10.4 out of 200 cells with an even distribution, nuclear aggregates, perinuclear aggregates, and cytosolic aggregates respectively. The same statistical test was used to yield p-values of 0.003 for the even distribution, 0.016 for the nuclear aggregates, 0.002 for the perinuclear aggregates, and 0.048 for the cytosolic aggregates. Even with the additional category for describing the aggregate phenotypes, the wild type TR showed nuclear, perinuclear, and cytosolic aggregates significantly more when co-transfected with the mutant TR than when transfected alone. Wild type TR distributed significantly less evenly in the presence of the increased expression of mutant TR as well. Furthermore the percentage of cells with wild type TR aggregates shows a clear increase from a 1:1 to a 1:2 wild type to mutant co-expression, indicating that the dominant-negative effect increases with increasing amounts of mutant TR.
**Figure 11:** Perinuclear aggregates. A) Merged image B) DAPI C) GFP- TRα1 (K74E, A264V) D) mCherry- TRα1
**Figure 12:** Analysis of the phenotypes of wild type TR in cells transfected with an increase of TRα1 (K74E, A264V) plasmid. Bars indicate SEM.

**Figure 13:** Analysis of GFP-TRα1 (K74E, A264V) as a control to the analysis of wild type distribution in response to an increase in mutant plasmid. Bars indicate SEM.
As before, the behavior of the mutant expressed with and without the wild type TR was also analyzed (Figure 13). Cells expressing only GFP-TRα1 (K74E, A264V) had 138.1 out of 200 cells with an even distribution, 16.6 cells with nuclear aggregates, 18 cells with perinuclear aggregates, and 27.3 cells with cytosolic aggregates on average. Cells expressing GFP-TRα1 (K74E, A264V) with mCherry-TRα1 exhibited an average of 136.5 out of 200 cells with an even distribution, 15.5 cells with nuclear aggregates, 16.95 cells with perinuclear aggregates, and 29.125 cells with cytosolic aggregates. P-values of 0.85, 0.71, 0.71, and 0.77 were obtained for the categories of even distribution, nuclear aggregates, perinuclear aggregates, and cytosolic aggregates respectively, indicating no significant differences between the mutant TR co-transfected with the wild type and the mutant TR transfected alone. Therefore the differences between wild type distributions under the previously described conditions were not likely to have been affected by an abnormal increase in aggregation from the co-transfected mutants.

The high frequency of perinuclear aggregation could indicate the beginnings of aggresome formation within the cell. Aggresomes are a cellular response to misfolded proteins linked to cancer and neurodegenerative diseases (Johnston et al., 1998). The viral oncogenic TR, ν-ErbA, displays dynamic trafficking to aggresomes, contributing to its dominant negative activity (Bondzi et al. 2011). While the specific foci associated with aggresomes were seen less frequently, longer periods of mutant expression could allow time for the coalescing of the individual aggregates into aggresomes. Aggresomes are further characterized by recruitment of chaperones and proteasomes and the breakdown and restructuring of the cytoskeletal protein vimentin that area (Johnston et al., 1998). Without these additional verification methods, the presence of aggresomes cannot be confirmed. Nevertheless, these data provide additional support
for the potential of dominant-negative sequestration of wild-type TR into aggregates by TRα1 (K74E, A264V).

Discussion

**TRα1 (K74E, A264V) displays nuclear and cytosolic aggregation**

Previously, investigations into the HCC mutant TRα1 (K74E, A264V) have focused on its altered target gene repertoire and its decreased sensitivity to T₃ (Chan and Privalsky, 2009; Chan and Privalsky, 2010). Studies using the model oncogenic TR, v-ErbA, have introduced new ways in which mutant TRs can impede normal cellular functioning, notably through mislocalization and aggregate formation (Bonamy et al., 2006). These experiments determined that TRα1 (K74E, A264V) displays an altered localization by way of aggregate formation and a capability to induce wild type TRα1 aggregate formation.

Protein aggregation is a process in which monomer peptides or proteins self-associate into non-native and less soluble structures (Bartolini and Andrisano, 2010). Aggregates are generally associated with misfolded proteins, so the significant levels of aggregation in the mutant TR suggest one or both of the mutations may have changed the conformation of either or both of the mutation-containing domains. This could increase the likelihood of overall misfolding within the cell. Given the often complete aggregation within cells shown here, misfolded TRs might interact with other TRs to promote further misfolding. Additional research needs to be conducted to expound the mechanisms mediating nuclear and cytosolic aggregation and the potential significance of this process in cancer cells *in vivo.*
One question which arises from these results is whether the localization of the nuclear aggregates is random or more directed. GFP 170*, a nuclear aggregate forming protein, is known to alter the organization of subnuclear domains containing the promyelocytic leukemia protein (PML) proteins known as PML bodies (Fu et al, 2005). Subnuclear domains are part of the organizational structure of the nucleus, providing centers of ribosome biogenesis in the nucleolus, RNA splicing in nuclear speckles, nuclear retention of specific RNA molecules which can associate with the nucleolus in paraspeckles, potential regulation of splicing machinery components in Cajal bodies, and potential RNA metabolism in PML bodies (Mitrea and Kriwacki, 2016; Spector and Lamond, 2011; Fox et al., 2002). The specific and full range of function for most of these remains unknown. Preliminary studies were designed to determine potential colocalization with the previously characterized nuclear aggregate forming GFP 170*, though the results only showed a modest correlation which could simply be indicative of the largely nuclear localization shared between the two proteins. Therefore, the question of whether the aggregates formed by the mutant TR also localize to and affect the architecture of subnuclear domains such as PML bodies has yet to be determined.

TRα1 (K74E, A264V) induces aggregate formation in TRα1

TRα1 generally distributes evenly with a mostly nuclear distribution. When expressed with TRα1 (K74E, A264V), a significant portion of the cells displayed visible aggregates above the background distribution of fluorescence. This phenotype increases with increased amounts of TRα1 (K74E, A264V)-containing plasmid, providing strong evidence that the mutant directly effects this behavior in its wild type counterpart. This is significant because each cell has two
copies of the THRA gene, so dominant-negative activity of the mutant TR will likewise affect the wild type TR in vivo.

Sequestration of wild type TR in aggregates may contribute to the dominant-negative activity of TRα1 (K74E, A264V). Although the induced aggregates would appear to colocalize with the mutant aggregates, the epifluorescent microscope can only give two-dimensional images. Though it is unlikely, fluorescent proteins aggregates that appear to coincide may simply be along the same z-axis. Therefore, other methods such as confocal microscopy would be needed to verify the colocalization suggested in this study.

The oncogenic TR model, v-ErbA, was shown to sequester a subset of TRα in aggresomes (Bondzi et al., 2011), so with the formation of aggregates in TRα1 (K74E, A264V), there is a question of whether aggresomes will form under certain conditions and whether that could also play a role in the pathogenesis of this mutant. Aggregates may coalesce to form aggresomes through motor-dependent movement on microtubules (Fu et al. 2005). The formation of a cytosolic aggresome is associated with the collapse of the intermediate filament vimentin, involved with the cytoskeletal structure of the cell. Aggresomes have foci near the nucleus at centrosomes, and vimentin forms a cytoskeletal cage around them to contain the misfolded proteins and mitigate the toxic effect on normal cell functioning. Chaperones and proteases are recruited to the structure, though it remains unclear whether aggresomes are cytoprotective in that deleterious proteins are sequestered or pathogenic as they are frequently associated with disease (Bondzi et al. 2011). Although the increase of perinuclear aggregates with the transfection of increased amounts of mutant TR plasmid could potentially indicate the result of
microtubule-dependent movement of aggregates towards the centrosomes, all of these cellular events would need to be investigated before the confirmation of this structure. The role of aggresomes in v-ErbA pathogenesis has been determined, but thus far any relevance to TRα1 (K74E, A264V) is purely speculative.

**Future Directions**

Initial results strongly support the partial colocalization of TRα1 with TRα1 (K74E, A264V) aggregates, and they merit a more sophisticated method of analysis with tools such as confocal microscopy or fluorescence resonance energy transfer (FRET). Confocal microscopy can reveal the three-dimensional structure of a specimen, selectively collecting light from a thin optical section at the plane of focus so that no out-of-focus fluorescence from different depths within the cell interferes with the image (Smith, 2008). With confocal microscopy, overlap of fluorescence at an exact point on a focal plane would be more easily seen and therefore colocalization would be more easily determined. FRET is a distance-dependent physical process in which an excited fluorophore (the donor) transfers energy to another fluorophore (the acceptor) (Sekar and Periasamy, 2003). During the transfer, some energy is lost, and the fluorescence emitted is of a different wavelength and therefore a different color than what would be emitted from the donor fluorophore. Interactions between proteins can be measured if one protein is labeled with a donor fluorophore and the other is labeled with an acceptor fluorophore. When a FRET event occurs, the fluorescent intensity of the donor will be reduced and that of the acceptor will be increased; measuring these signal changes could allow for the measurement of colocalization between proteins such as TRα1 and TRα1 (K74E, A264V) within aggregates.
In order to examine whether TRα1 (K74E, A264V) localizes to a subnuclear domain, proteins characteristic of certain nuclear domains, such as PML protein for PML bodies, could be immunostained and then analyzed for colocalization with the GFP-tagged mutant. Confocal microscopy, or another method for colocalization analysis, would suit this effort as well. Additionally, the domain protein and TRα1 (K74E, A264V) could be labeled with a donor/acceptor pair for FRET. These methods would provide more definitive answers as to whether there are specific foci for nuclear aggregate formation.

Furthermore, to explore whether potential sequestration of TRα1 in aggresomes is a factor in HCC, conditions could be altered to promote aggresome formation. Allowing for increasing amounts time of between transfection and fixation could promote a greater degree of microtubule-dependent of aggregates into aggresomes. Additionally, as HCC frequently develops from cirrhotic livers, cells could be exposed to similar kinds of stressors that would better replicate conditions in HCC tumors. To further approach in vivo behavior, it may be more beneficial to use hepatocytes for these studies. This can be paired with visualization via immunostaining (using fluorescently-labeled antibodies specific for the protein of interest) the mutant TR as opposed to overexpression studies, using gene editing to introduce the needed mutations. This would ensure that any resulting altered localization occurs under normal expression levels and is not simply due to an excess of protein. Using these additional measures could better elucidate how TRα1 (K74E, A264V) behaves in the cancerous liver.

Results from these studies will spark new questions and bring new answers to advance the understanding of the many ways TRα1 (K74E, A264V) interferes with normal cellular
functioning to promote oncogenesis, paving the way for better treatment options for individuals with HCC and other cancers involving mutant TRs.

References


Lee Y., Mahdavi V.(1993) The D domain of the thyroid hormone receptor α1 specifies positive and negative transcriptional regulation functions. J. Biol. Chem. 268, 2021–2028


Appendix A

Transfection Protocol

After the cells have been trypsinized, add 15 μl of the cell suspension to a hemacytometer to determine the cell number, and add ~2.5 x10^5 cells per well to a 6-well plate with glass coverslips. Incubate the cells at 37°C for ~24 hours.

In a biosafety cabinet, dilute target amount of plasmid DNA with Opti-MEM (reduced serum media) for a total volume of 250 μl in 6 separate tubes. In a different tube, add 1476 μl Opti-MEM, then add 24 μl for a total volume of 1500 μl. Incubate the solutions for 5 minutes at room temperature.

Add 250 μl Lipofectamine-Opti-MEM to each of the DNA-Opti-MEM solutions for a total volume of 500 μl. Mix gently then let the solution to sit at room temperature for 20 minutes to allow formation of DNA-liposome complexes.

Add the DNA-liposome complexes to each of the prepared wells. Mix gently, and incubate the cells plus complexes for 6-8 hours at 37°C.

Replace the medium with fresh, complete medium.

Appendix B

D-PBS

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.10 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.10 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.00 g</td>
</tr>
<tr>
<td>Na₂HPO₄•7H₂O</td>
<td>1.08 g</td>
</tr>
</tbody>
</table>

Add ddH₂O to 500ml, autoclave, and store at room temperature.