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Acetylation Controls the Subcellular Localization of Thyroid Hormone Receptor α1

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By

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Abstract

Thyroid hormone is produced by the thyroid gland through the hypothalamic-pituitary-thyroid axis. It is critical for growth, development, and homeostasis, and its action is mediated by the thyroid hormone receptor alpha1 (TRα1). TRα1 is a transcription factor that activates or represses target genes in response to thyroid hormone. Although primarily localized to the nucleus at steady state, TRα1 shuttles rapidly between the nucleus and cytosol. This thesis research focused on elucidating how post-translational modification of TRα1 modulates its nucleocytoplasmic transport. TRα1 is known to be acetylated at lysine residues 128, 132, and 134. In order to determine whether acetylation of TRα1 plays a role in regulating nucleocytoplasmic transport, expression plasmids for GFP or mCherry-tagged TRα1 mutants that mimic acetylation (lysine to glutamine substitutions) and nonacetylation (lysine to arginine substitutions) were constructed. Fluorescence microscopy was used to determine the nuclear/cytosolic (N/C) ratio of the fusion proteins in transfected cells by measuring fluorescence intensity. N/C data showed that the TRα1 nonacetylation mimic and wild-type TRα1 both have a primarily nuclear localization, and that intracellular distribution patterns of the TRα1 nonacetylation mimic and wild-type TRα1 were not hormone dependent. Furthermore, when co-transfected the presence of the TRα1 nonacetylation mimic did not change wild-type TRα1 localization. In contrast, the TRα1 acetylation mimic showed a lower N/C ratio compared to wild-type TRα1, indicating a significant decrease in nuclear localization. Taken together, these data suggest that interactions between TRα1 and transport factors may depend on electrostatic interactions. These findings will extend understanding of the role of post-translational modifications in regulating the fine balance between nuclear import, export, and nuclear retention, and how this interplays with TRα1 transcriptional regulation.

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Special thanks goes to the members of Allison Lab. They all played an important part in making lab a fun and exciting experience. I would specifically like to thank Masters Student Dylan Zhang for his help and guidance in helping me improve my skills as a researcher, and for his friendship and support throughout my years in Allison Lab. Finally, without the love and support of my parents and siblings, I would not be where I am today.

General Introduction

In a typical eukaryotic cell, there is a distinct separation between nuclear genomic material and other compartments in its aqueous environment. This separation necessitates the highly controlled bidirectional traffic of macromolecular molecules such as RNA and regulatory proteins (Terry et al., 2007). Elucidating the complex pathway of nuclear entry and exit has been the goal of researchers for the past several decades. The major goal of this thesis work was to determine the effect of post-translational modification of the thyroid hormone receptor alpha1 (TRα1) by acetylation on the receptor's complex transport pathway. TRα1 is a transcription factor that activates or represses target genes in response to thyroid hormone. The following introduction provides background information on the thyroid hormone receptor (TR) and presents an historical context to the field of nucleocytoplasmic transport. In addition, the methods and objectives of this thesis research are explained.

Thyroid Hormone

Thyroid hormone affects many processes such as the growth, development, and metabolism of virtually all tissues within our body (Chen et al., 2013; Samuels & Tsai, 1973). The hormone is exclusively produced through a feedback loop that includes the hypothalamus, pituitary, and thyroid gland, commonly referred to as the hypothalamic-pituitary-thyroid (HPT) axis (Vella et al., 2014; Yen et al., 2001). The hypothalamus is also responsible for regulating the production of all other hormones within our bodies. The HPT axis essentially involves a series of signal transduction cascades, where a

signal sent from the hypothalamus “master gland” eventually arrives at the thyroid gland.

The thyroid hormone is produced in two forms, and the structure of each form is directly tied to its function. Thyroxine (T_4) contains four iodine molecules and triiodothyronine (T_3) contains three. T_4 is the major form of thyroid hormone that is produced by the thyroid gland, and T_3 is the active form generally synthesized from the deiodination of T_4 (Gnocchi et al., 2016). T_4 is not directly involved in mediating gene expression; instead, it indirectly influences gene expression by cell signaling. In contrast, because T_3 binds to TR within the cell it is directly involved in mediating gene expression.

The intricate balance between thyroid hormone production, conversion from T_4 to T_3, and binding of T_3 to TR is critical for receptor mediated gene expression; as a result, the dysregulation of this process leads to diseases such as: resistance to thyroid hormone, cancer, dwarfism, and metabolic disorders (Cheng, 2005). These diseases are a motivation for this thesis work. The basic research on the transport pathway of TR specifically dealing with acetylation, a post-translational modification, helps to breakdown the complex interactions of TR with other proteins and thyroid hormone.

Thyroid Hormone Receptor

Structure

In humans the two main isoforms of TR, alpha (α) and beta (β), are transcribed by two genes on chromosomes 17 and 3, respectively (Ruiz-Llorente et al., 2010). After post-transcriptional processing the transcripts are then transported out of the nucleus to

be translated by ribosomes into a functional TR isoform. The overall structure of TR consists of modular domains lettered A/B, C, D, E, and F. These letters represent the evolutionarily conserved domains of nuclear receptors: activation function-1, DNA binding domain, hinge region, dimerization/ligand binding, and activation function-2, respectively (Pawlak et al., 2012). TRα contains two nuclear localization signals (NLSs). The stronger of the two is in its hinge region and the weaker is in its A/B domain. TRβ contains only one NLS in its hinge region (Mavinakere et al., 2012). Members of the importin family of karyopherins, specifically importin 7, importin β1, and adapter importin α1 recognize the NLSs and directly mediate the nuclear import of TRα (Roggero et al., 2016). The cycle of transcription, transport of the mRNA out of the nucleus, translation of TR mRNA, and then import of TR into the nucleus begins anew.

Regulatory Activity

Once in the nucleus, TR regulates gene expression responsible for cellular functions such as differentiation, development and metabolism (Lopez et al., 1993; Ruiz-Llorente et al., 2011; Suh et al., 2013; Wagner et al., 1995; Xing et al., 2016). Transcriptional regulation is conducted through the interactions with coactivator or corepressor complexes that modify DNA to facilitate or inhibit the activity of transcription factors (Green and Han, 2011).). Many studies show that thyroid hormone plays an important role in TRα1 transcriptional regulation (Bernal and Morte, 2013; Brent, 2012; Fondell, 2013; Grøntved et al., 2015; Yuan et al., 2013). In the absence of thyroid hormone, expression of thyroid hormone related genes is usually silenced (Brent, 2012; Zhang et al., 2000). There are some instances of TR's regulatory activity in which gene

expression is activated in the absence of thyroid hormone (Aranda and Pascual, 2001; Ayers et al., 2014).

Nucleocytoplasmic transport

The field of nucleocytoplasmic transport has been rapidly growing since the 1960s; since then there have been many landmark discoveries that have helped to further advance the field. This review discusses some of these landmark studies, focusing in particular on: assembly/disassembly of the karyopherin complex, transport factors, and the binding specificity of transport factors to cargo.

Background

Nucleocytoplasmic transport is an essential cellular activity that occurs through nuclear pore complexes (NPCs) residing in the double membrane of the nuclear envelope (Dickmanns et al., 2015). The NPC is composed of nucleoporins (nups); each nup is composed of amino acids linked together by peptide bonds. Together the nups form a basket-like structure that binds transport factors. Nups are typically made up of repeat regions of amino acids such as phenylalanine (F), glycine (G), and leucine (L). Nuclear import and export pathways are mediated by a family of transport factors known as importins or exportins, collectively known as karyopherins. In order to be targeted to the nucleus, proteins must contain a specific amino acid sequence called a nuclear localization signal (NLS). NLS-containing proteins interact with members of the importin family of receptors by either a monopartite (consisting of one part) or bipartite (consisting of two parts) NLS, and nuclear export signal (NES) containing proteins

interact with members of the exportin family of receptors by either a monopartite or a bipartite NES (Allison, 2012).

Due to the NPC, the passage of proteins into and out of the nucleus is very tightly regulated. This tightly regulated transport of proteins through the NPC has been recognized as a crucial step in many cellular processes (Hodel et al., 2001). Some of these cellular processes include: mitosis and gene expression (Matsuura and Stewart, 2004); communication between neurons (Panayotis et al., 2015); regulation of the cell cycle and proliferation of normal and malignant cells (Gravine et al., 2014). By the late twentieth century, it was already established that nups function as docking proteins for karyopherin-mediated binding of substrates in a nuclear import/export pathway across the NPC (Radu et al., 1995).

Karyopherin Complex Assembly and Disassembly

In a landmark study (Rexach and Blobel, 1992), researchers sought to understand the interactions that take place between the mobile phase of transport (transport factors and substrate) and the stationary phase (nucleoporins). They first found, using a solution binding assay, that the karyopherin heterodimer (kap60 and kap95) bound to the FXFG (X indicates small amino acid residues such as serine, glycine or alanine; Bayliss et al., 2002) repeat region of Nup1 and 2, but not to the GLFG repeat region of Nup57 or 145.

Rexach and Blobel (1992) also examined, once they established that kap60 and kap95 were able to bind to the NPC, the assembly of the kap60 and kap95 heterodimer to an NLS-containing protein. They found that kap60 monomers bound to the NLS

protein and that kap95 monomers did not. Furthermore, the addition of increasing amounts of kap95 resulted in an increase in the binding of kap60 to the NLS protein. Taken together, these results established that the karyopherin heterodimer-GST-NLS complex binds to the FXFG repeat region in a fashion that stimulates the release of the NLS protein from kap60. This finding led to the question of how the dissociation of the karyopherin heterodimer complex occurs with the interaction of the NPC FXFG repeat region. The researchers sought to determine whether Ran, in its bound and unbound guanine phosphate forms, had a distinct effect on the dissociation of karyopherins from the FXFG repeat region. They found that Ran in its GTP bound form was able to cause the dissociation of both karyopherin subunits from the repeat region. Using Ran bound GMPPcP they were able to establish that GTP hydrolysis was not required for this reaction. Ran bound GMPPcP is a guanine nucleotide analog that was used as a screen to test for GTPase activity as a requirement for dissociation of the cargo complex.

The next question asked by Rexach and Blobel (1992) dealt with where RanGTP had to bind in order for dissociation of the karyopherin subunits from the FXFG repeat region. They found that RanGTP disrupts the karyopherin heterodimer by binding to karyopherin β. Taken together, their findings revealed several association-dissociation reactions that occur between nucleoporin FXFG repeat regions, transport factors, and NLS proteins.

Another landmark study helped to elucidate the structural basis for the assembly of a nuclear export complex (Matsuura and Stewart, 2001). The researchers' goal was to address the opposite function of RanGTP in nuclear export, specifically, why was

complex formation required to bind rather than release cargo in the nucleus? They addressed this question by establishing the crystal structure of the nuclear export complex formed by exportin Cse1P complexed with its cargo (kap60p) and RanGTP. They found that in the complex Cse1P coils around both RanGTP and kap60, stabilizing the RanGTP-state and clamping the kap60 importin-β-binding domain, ensuring that only cargo-free kap60p is exported.

These early studies helped to establish the basis for the assembly and disassembly of transport factors via the Ran gradient. They also helped to distill the complex steps of nucleocytoplasmic transport into simple stepwise reactions.

Transport Factors

In an important study to the field of nucleocytoplasmic transport, researchers painted a more elaborate picture of transport factors (Bonifaci et al., 1997). Their findings suggested that import of nuclear proteins occurs by multiple pathways, and that proteins are directed into these pathways by distinct NLSs. During the time this paper was published, the transport factors found in yeast such as kap60p (karyopherin α) and kap95p (karyopherin β) were used as substitutes for their mammalian homologs. Studies in yeast had revealed the existence of three proteins both structurally and functionally related to kap95p; as a result, they were classified as members of the yeast karyopherin β family. All four yeast β karyopherins [kap95p, kap104p, Pse1p, and kap123p] had been shown to serve as nuclear protein import transport factors. These yeast karyopherins help to elucidate the transport pathways of their mammalian homologs.

A study that illustrates characterization of transport factors, specifically those found in the importin β superfamily, was conducted by Jakel et al. (1999). In this study, the researchers investigated the nuclear import of linker histone H1 and found that two receptors, importin β (Impβ) and importin 7 (Imp7 or RanBP7), play a critical role in this process. At the time this study was conducted, it was still unclear as to whether RanGTP-binding to Imp7 was needed to complete NPC passage or just to release the cargo from Imp7 into the nucleus. The model proposed by Jakel et al. is summarized in FIG.1.

Chaves and Jonathan (2011) took a different approach to the study of nuclear transport by asking the question of whether mutations in a cargo proteins' NLS was sufficient to switch its cognate karyopherin. Even though recent advances in the field suggested the likelihood that each of the karyopherins recognizes its own type of NLS, this step was still poorly understood (Bonifaci et al., 1997). Thus, Chaves and Jonathan (2011) sought to provide evidence that the affinity of the karyopherin/signal interaction was a critical factor in determining transport efficiency and selectivity by characterizing the NLS of the yeast homolog of the mammalian La protein, Lhp1. The La protein plays a major role in a variety of processes such as: stabilization of RNA structure, retention of small RNAs in the nucleus, facilitation of RNP (ribonucleoprotein) assembly, and accurate tRNA processing (Chaves and Jonathan, 2011). The Lhp1 NLS consists of 112 residues and is targeted to the nucleus in a kap108-dependent manner (Chaves and Jonathan, 2011). They found that the mutation of three of the 275 residues in full-length Lhp1 alters its import pathway to a kap121-dependent process; in addition, wild-type function was not retained by the mutant. Chaves and Jonathan (2011) proposed

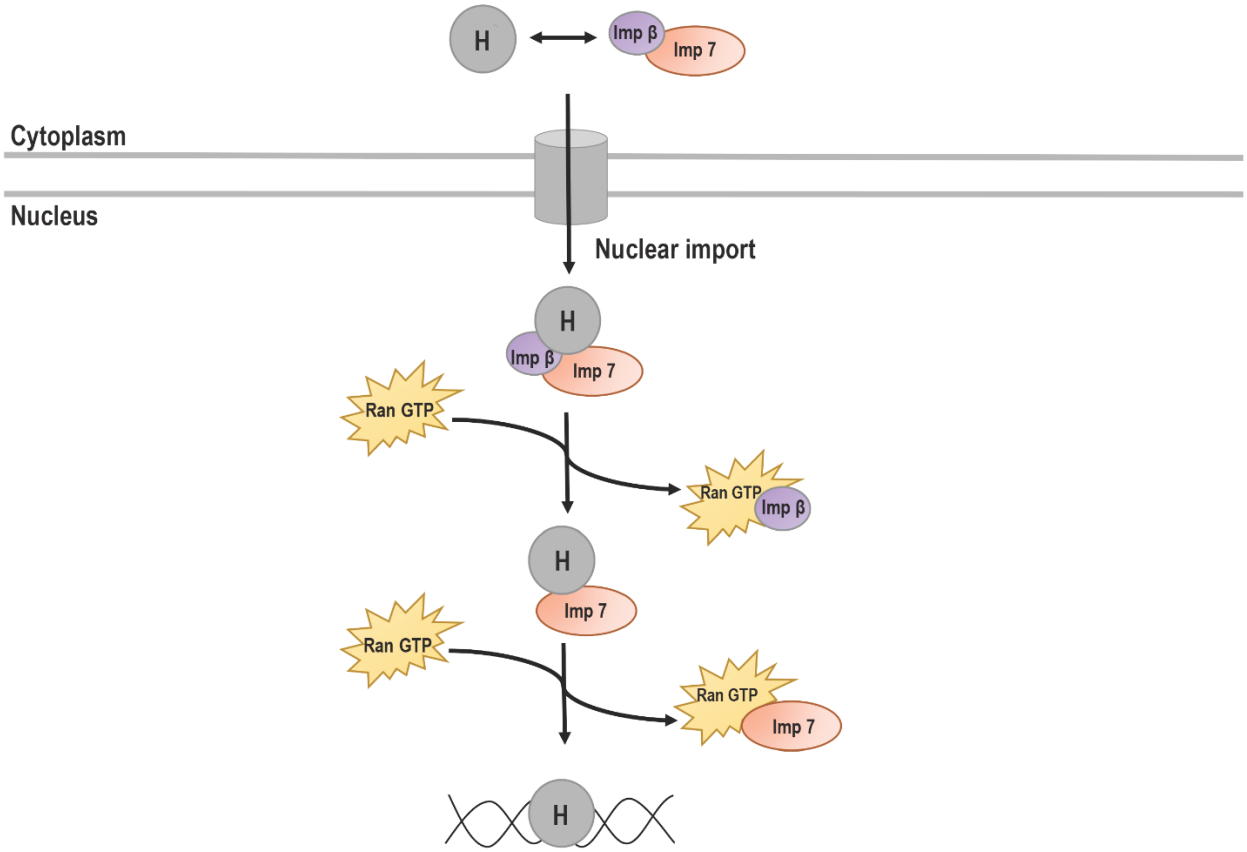


Figure 1. Diagram of histone (H) import into the nucleus.
Histones are one of the most abundant import substrates, and during S-phase in HeLa cells, approximately one histone molecule per second is imported into the nucleus. This diagram shows a trimeric cargo complex assembled in the cytoplasm. The complex translocates through the NPC into the nucleus, and the disassembly of the trimeric complex results from RanGTP binding. The histone is then free to bind DNA.

Adapted from Jakel et al., 1999

that kap121 functions as a chaperone, one that can serve as a genetic buffer, to transport mutated proteins to the nucleus.

Binding Specificity

There has been intense research effort put into the analysis of the recognition of NLSs by import factors since the first NLS peptides were discovered in the 1980s (Soniat and Chook, 2015). In a key study by Conti et al. (1998), researchers used X-ray crystallography to analyze the recognition of a NLS by karyopherin α 50 (kapα50). They found that the structure of kapα50 contained ten tandem armadillo (ARM) repeats, organized in a right-handed superhelix of helices. Their work in analyzing the overall structure of kapα50 revealed the determinants of NLS specificity and suggests a model for the recognition of bipartite NLSs.

Recently, there has been a drive toward understanding nucleocytoplasmic transport in terms of a charge-driven mechanism. With the development of better technology and higher microscopic resolution, researchers are now able to uncover the mechanistic fundamentals for nuclear transport of charged substrates through the NPC. Goryaynov and Yang (2014) sought to examine the role of molecular surface charge, compared to the influence of molecular size and specific signal, in nucleocytoplasmic transport. They found that electrostatic interaction between negative surface charges on transiting molecules and the positively charged FXFG (FG) nups, although enhancing their probability of binding to the NPC, does not usually play a dominant role in nucleocytoplasmic transport (FIG. 2).

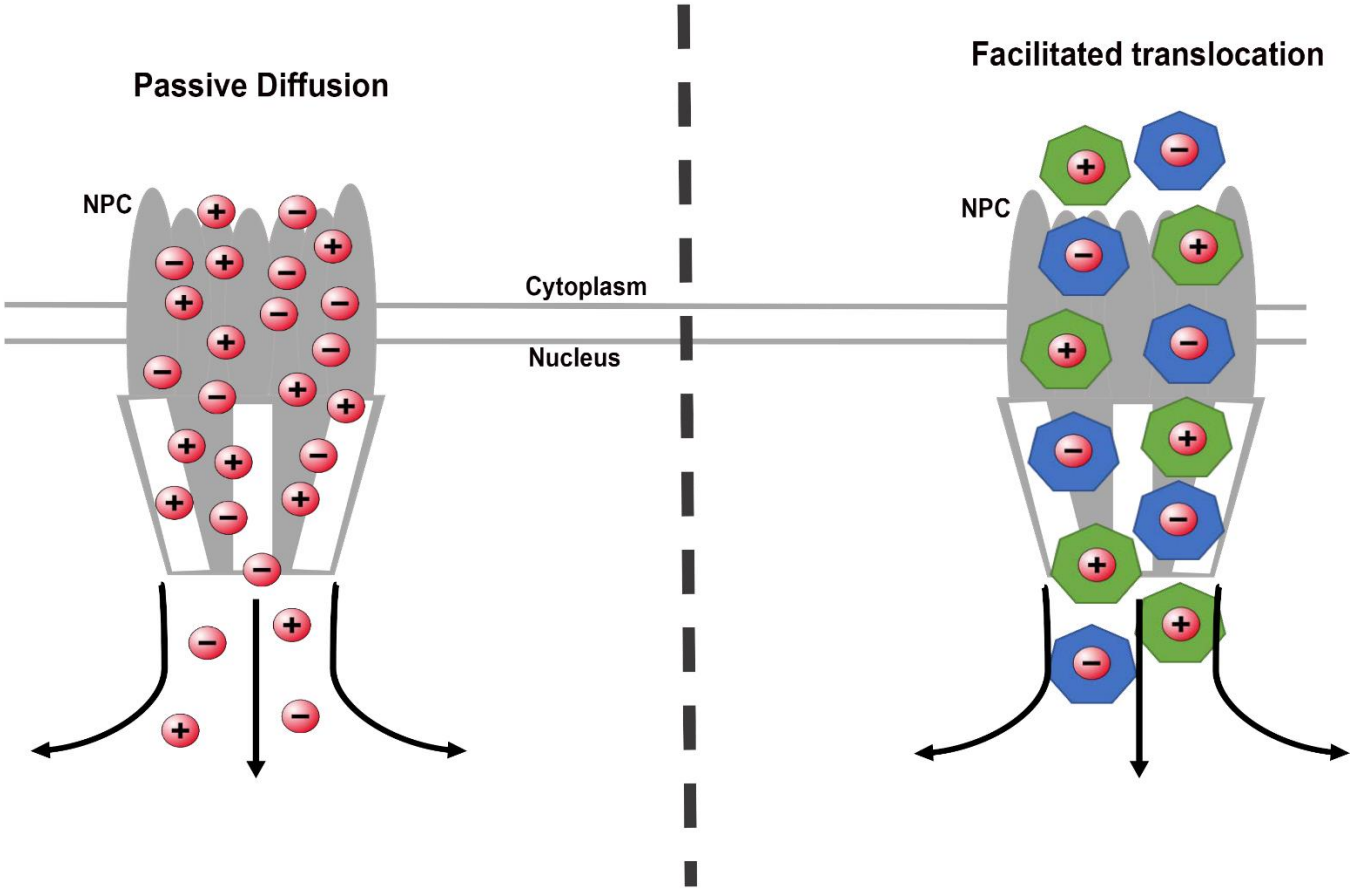


Figure 2. Transport routes of small passively diffusing molecules and transiting cargo complexes.
A selective barrier formed by the NPC allowing for the passive diffusion of small signal-independent molecules, and transport-receptor facilitated translocation of signal-dependent cargo molecules.

Adapted from Goryaynov and Yang, 2014

With respect to nucleocytoplasmic transport in neurons, Lever et al. (2015) asked the question of whether there is an ‘importin code’ in neuronal transport from synapse-to-nucleus. To date, there has been very little research on importin function in neurites. In their opinion paper, the researchers point out the reductionist nature of research regarding nuclear import, and propose that nuclear import is much more complicated than commonly thought. They specifically point out the limitations of the classical importin pathway, namely that as non-canonical importin functions are emerging, the role of importins in transport cargo specificity is likely being underestimated (Lever et al., 2015). As a result, in an attempt to provide an explanation for the regulation of which synaptic proteins are to be transported into the nucleus, they hypothesize the existence of an ‘importin code’ for neurons that acts as a highly specific system capable of selecting more than 200 synaptic proteins for activity-dependent nucleocytoplasmic shuttling in accordance with the current environment (FIG.3).

Future Prospects

The field of nucleocytoplasmic transport has made many key advances since the mid-1900s, from the identification of a nuclear protein containing a nuclear localization signal, to the stepwise characterization of the localization signal and its resulting cognate karyopherin. With the establishment of the basics of nucleocytoplasmic transport, there have been recent trends towards application of this information to target human diseases such as cancer and subvert viral infections (Cautain et al., 2015). The field of nucleocytoplasmic transport has come a long way in the past several years, and

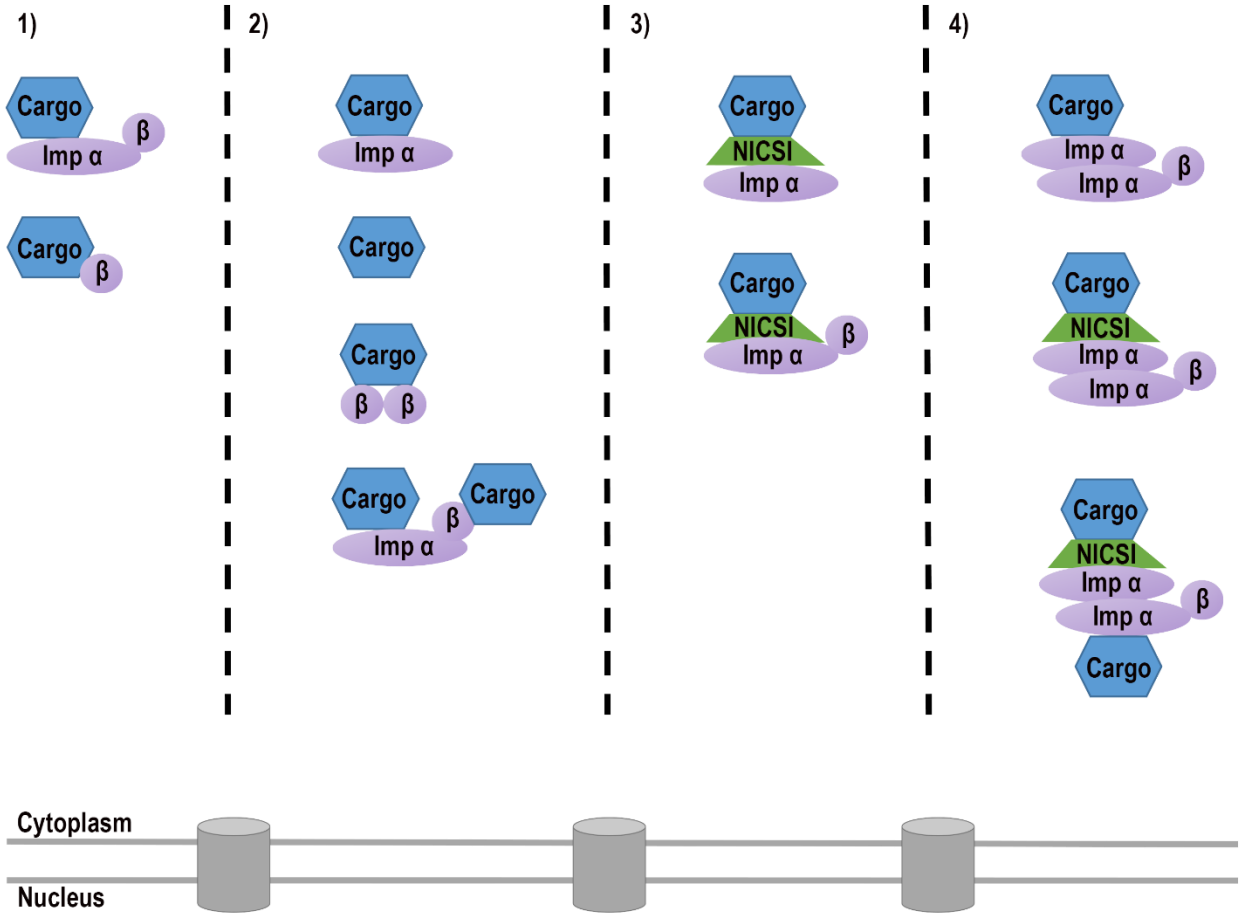


Figure 3. Canonical and non-canonical importin structures and the Importin Code. A depiction of the proposed 'importin code'. 1) Canonical importin cargo complex. 2) Caveats to the canonical description of complex formation. Importin α can mediate cargo transport alone, cargos can execute their own nuclear import, importin β can mediate transport, and importins can carry two cargos. 3) Proposed importin code incorporating non-importin family member cargo-specific importins (NICSIs). 4) A highly speculative model of importin code complex formation. The importin code could be best described as unique importin combinations that provide tight control over synaptic protein selection for nuclear import and the governance of cellular phenotypes, much like how a barcode can read and decode a specific item into the readout of a price. There are estimated to be approximately 2700 synaptic proteins, and out of these approximately 10% possess bona fide NLSs. Some of these synaptic proteins include: tau, contactin-associated protein 1 (Caspr1), disrupted in schizophrenia 1 (DISC1), and others (consulted from R&D Systems a biotechne brand, 2015).

Adapted from Lever et al., 2015

the continued drive to find new discoveries has paved the way to better understand the fundamentals of processes that take place within the cell.

Post-translational modification

There are more than 200 types of both rare and common post-translational modifications (PTMs) that have been found to occur in proteins (Azevedo and Saiardi, 2016a). PTMs play a significant role in the regulation of proteins by altering structure, enzymatic activity, stability or degradation, subcellular localization, protein-protein interactions, and diverse cell signaling (Cui et al., 2004; Dohmen, 2004; Hock et al., 2010; Ito et al., 2007; Johnson, 2004; Lee et al., 2016; Li et al., 2002; Meek and Knippschild, 2003; Pinceti et al., 2015; Tan et al., 2014; Vierstra, 2012). Many amino acid side chains such as serine, threonine, and tyrosine are post-translationally modified; however, lysine residues are targeted by an extremely high number of PTMs including methylation, ubiquitination, sumoylation, and acetylation (Azevedo and Saiardi, 2016b).

Acetylation

Acetylation of histones and other proteins and the functional consequences of acetylation have been topics of scientific interest for the past 50 years (Simon et al., 2016; Yang et al., 2015). Since lysine acetylation was initially identified in histones, many lysine acetyltransferases and lysine deacetylases are often referred to as histone acetyltransferases and histone deacetylases (Choudhary et al., 2009; Kadiyala & Smith, 2014; Yang and Seto, 2008). Acetylation regulates the biological functions of many

proteins that play a role in cellular homeostasis. Furthermore, it has been proposed as an additional mechanism for regulating subcellular localization (Bannister et al., 2000; Bonaldi et al., 2003; Gay et al., 2003; Madison et al., 2002; Santos-Rosa et al., 2003; Soutoglou et al., 2000; Spilianakis et al., 2000).

The acetylation of lysine residues within proteins takes place on the epsilon (ε) amino group (NH_3^+) of lysines. The addition of an acetyl group on lysines neutralizes the positive charge on the amino group and significantly impacts the electrostatic properties of the protein (Dancy and Cole, 2015; Glozak et al., 2005; Wang et al., 2010). Some nuclear receptors that have been shown to be acetylated include the androgen receptor (AR) (Fu et al., 2000), the estrogen receptor alpha (ERα) (Cui et al., 2004; Kim et al., 2006), TRβ (Lin et al., 2005), and TRα (Sanchez-Pacheco et al., 2009). The discovery that lysine residues could be acetylated in the AR led researchers to use amino acid sequence equivalency to determine corresponding amino acid motifs that contain lysine residues in other nuclear receptors. The acetylation residues are part of a lysine/arginine motif present in the hinge domain of several members of the nuclear receptor family. To characterize the effect of acetylation on TRα, Sanchez-Pacheco et al. (2009) mutated lysine residues at varies sites on TRα to determine the functional consequences of acetylation on TRα. The main conclusion reached was that the lysine amino acid residues at sites 128, 132, and 134 in the hinge domain of TRα are essential for receptor acetylation, and that T_3 induced acetylation of wild-type TRα resulted in increased binding to DNA. However, a direct functional role of acetylation on ligand-dependent transcriptional activation or repression could not be established.

Subcloning

Genetic engineering is the use of molecular tools to manipulate DNA. Gene manipulation results in novel combinations of DNA and the techniques used to do this are referred to as recombinant DNA technology (Freeman, 2011). Subcloning is one of the main approaches in modern molecular biology, biochemistry, and protein engineering used to combine fragments of DNA to generate a single DNA molecule capable of autonomous replication in a given host cell (Ahmad et al., 1991; An et al., 1979; Biener et al., 2002; Erokhin et al., 2016; Hartley et al., 2000; Pham et al., 1998; Searle et al., 1984; Struhl et al., 2001; Zhang et al., 2015) (FIG.4). Enzymes known as restriction endonucleases (and DNA ligase) account for the success of subcloning (Fromme and Klingenspor, 2007).

Restriction Endonucleases

Restriction endonucleases are part of the restriction-modification systems that protect bacterial cells against foreign DNA (Mucke et al., 2003). These enzymes evolved from nonspecific endonucleases to cleave DNA sequences at highly specific target sites (Pingoud et al., 2014; Saravanan et al., 2008; Tóth et al., 2014). When used *in vitro* (in test tube), T4 DNA ligase from the T series of bacteriophages is used to recombine DNA fragments made by restriction endonucleases (Guo et al., 2016; Pusch et al., 1998; Rossi et al., 1997). The emergence of restriction endonucleases has helped in the advancement of human gene therapy techniques against diseases caused by mutations in DNA, such as X-linked severe combined immunodeficiency (X-linked SCID) (Flotte, 2007).

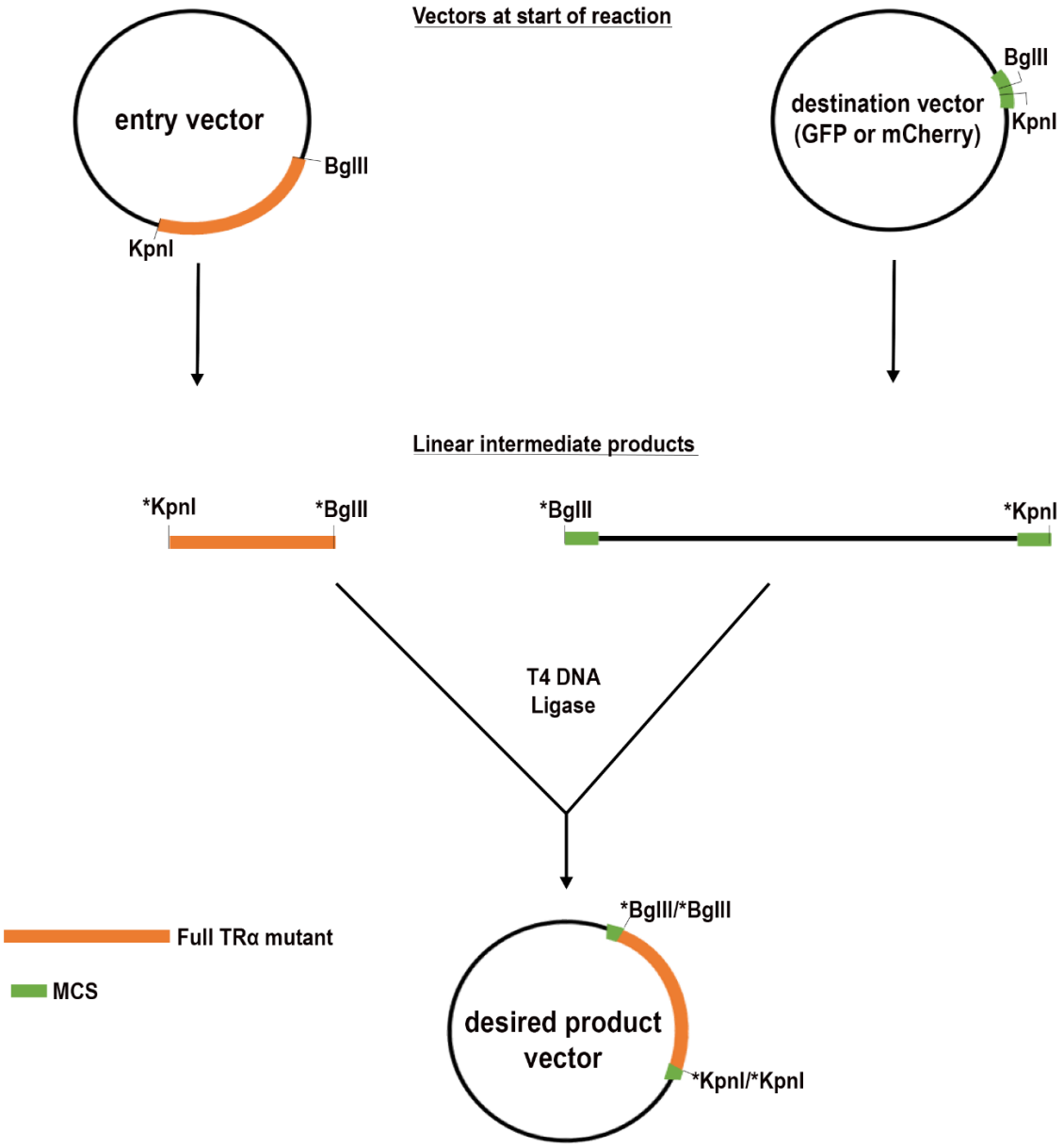


Figure 4. A schematic diagram of Subcloning
The upper region contains the two vectors the reaction starts with, i.e. the entry vector and the destination vector. The two vectors contain a recognition sequence for restriction endonucleases, in this case, BglII and KpnI. The destination vectors' recognition sequence is represented by the green box (MCS). MCS or, Multiple Cloning Sites, simply contain recognition sequences for many restriction endonucleases. By the step-wise enzymatic actions (arrows) of BglII and KpnI, these vectors are linearized to form linear intermediate products as shown. The asterisk (*) represents the cuts made by BglII and KpnI. These intermediates are subject to T4 DNA ligase activity and can then be ligated to yield the desired product vector.

Adapted from Fromme and Klingenspor, 2007

Thesis Objective

The fine balance between nuclear import, export, and nuclear retention has emerged as a critical control point for regulating TRα1 transcriptional activity (Mavinakere et al. 2012). In addition, Sanchez-Pacheco et al. (2009) determined that acetylation plays a role in TRα1's transcription activity. Because the amino acid lysine residues that are acetylated occur within the hinge domain of TRα1, there exists the possibility that acetylation may play a role in TRα1 transport activity. In this thesis research, the objective was to determine the effect of acetylation on TRα1's nucleocytoplasmic transport.

To test this a two-step process was used:

1. Construct TRα1 mutants that mimic TRα1 in its acetylated and nonacetylated states following standard practice (Yang et al., 2016) (FIG.5).
2. Analyze the subcellular localization of the mutants using fluorescence microscopy.

We hypothesized that the TRα1 nonacetylation mimic would have a predominantly nuclear localization and the TRα1 acetylation mimic would have a more cytosolic localization. This is because acetylation neutralizes the positive charge on lysine residues, and as the lysine residues K128, K132, and K134 are located within the hinge region NLS of TR, its affinity to bind importins would be reduced.

The results from this work will further our understanding of the mechanisms behind TRα1 nucleocytoplasmic transport and that of other nuclear receptors.

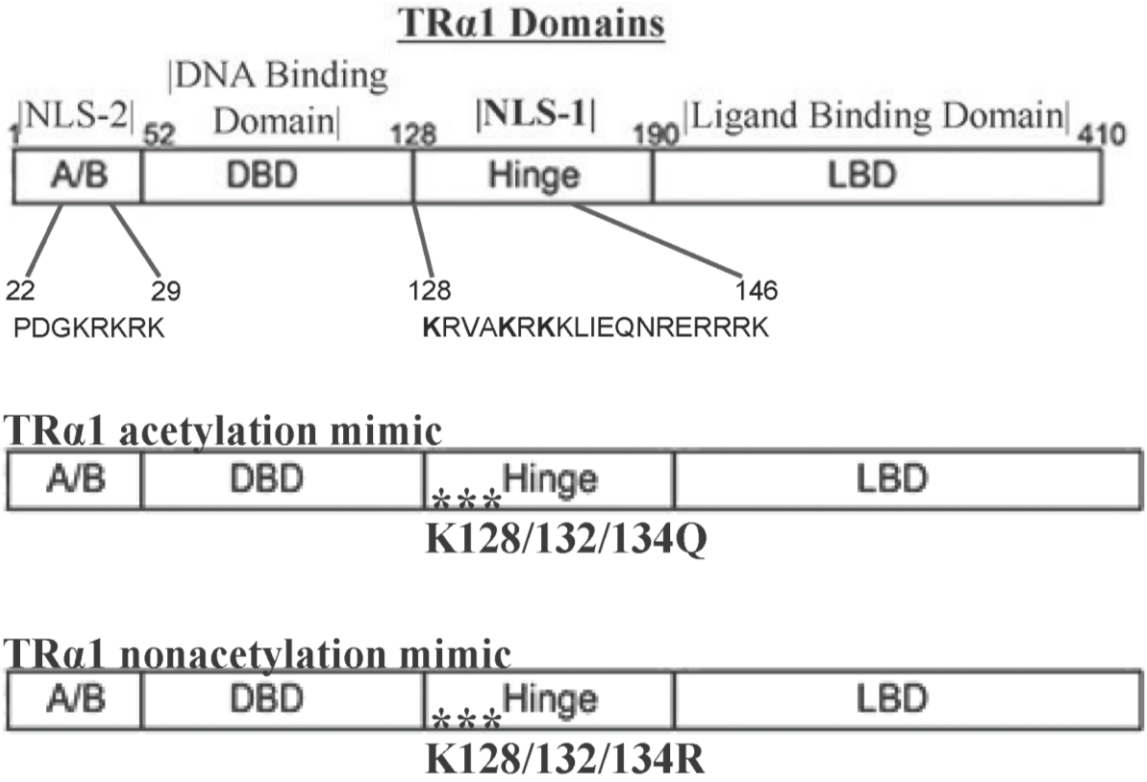


Figure 5. TRα1 mutant constructs
A diagram of the TRα1 domains, and the amino acid sequence of the NLS's in the Hinge and A/B domain. The top represents wild-type TRα1 and the bottom two represent the acetylation and nonacetylation mimics, respectively. Standard practice is to use the amino acid residue glutamine (Q) to mimic the effect of acetylation, and the amino acid residue arginine (R) to mimic the effect of nonacetylation.

Methods

Plasmids

The plasmid pGFP-TRα1 encodes a functional green fluorescent protein (GFP)-tagged rat TRα1 fusion protein (Bunn et al., 2001), and pmCherry-TRα1 encodes a red fluorescent protein (mCherry)-tagged rat TRα1 fusion protein. The expression vectors for enhanced GFP and mCherry, EGFP-C1 and EmCherry-C1, were obtained from Clontech Laboratories, Inc. (Mountain View, CA).

The mutant TRα1 acetylation mimic plasmid consisting of lysine (K) amino acid residues 128, 132, and 134 substituted for glutamine (Q) (TRα1-K128/132/134Q), and TRα1 nonacetylation mimic plasmid consisting of lysine (K) amino acid residues 128, 132, and 134 substituted for arginine (R) (TRα1-K128/132/134R) were designed and purchased from GeneArt, Inc. (Burlingame, CA). After purchase, 5 µg of the mutant plasmids were shipped from GeneArt to The Allison Lab, Department of Biology, College of William and Mary.

Subcloning

Mutant TRα1 acetylation mimic was subcloned into a mCherry expression vector and the mutant TRα1 nonacetylation mimic plasmid was subcloned into mCherry or GFP expression vectors. BglII and KpnI restriction digest enzymes obtained from New England Biolabs, Inc. (Ipswich, MA) were used for subcloning. The fluorescent tagged mutant TRα1 expression plasmids were then transformed into *E. coli*-DH5α subcloning efficiency bacteria obtained from New England Biolabs, and purified using ZymoPURE Plasmid Midiprep Kit, Zymo Research Corporation (Irvine, CA) per the manufacturer's

instructions. Plasmid concentration was determined by UV spectroscopy with the NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer, and final constructs were verified by gene sequencing on the departmental ABI PRISM® 3700 Genetic Analyzer.

Cell culture

HeLa cells (American Type Culture Collection [ATCC], # CCL-2) were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY) at 37ºC under 5% CO_2 and 98% humidity. Cells were grown to approximately 80% confluency before transient transfection procedures.

Transient transfection

HeLa cells were seeded at a density of 2-3 x 10^5 cells per well on 22 mm Coverslips for Cell Growth™ (Fisher Scientific, Pittsburgh, PA) in 6 well culture dishes. Twenty four hours post-seeding, cells were transfected with 2 µg of wild-type or mutant GFP or mCherry-TRα1 expression plasmid using Lipofectamine 2000 (Life Technologies). The transfection medium was replaced with fresh MEM containing 10% FBS at 6 hours post-transfection. Approximately 18 h later, cells were fixed in 3.7% formaldehyde, and coverslips were mounted with Fluoro-Gel II mounting medium (Electron Microscopy Sciences, Hatfield, PA) containing the DNA counter stain 4´,6-diamidino-2´-phenylindole dihydrochloride (DAPI, 0.5µg/ml). Cells were then analyzed for the cellular localization of wild-type or mutant GFP or mCherry-TRα1 by fluorescence microscopy.

For co-transfection, a similar experimental procedure was followed. However instead of 2 µg of plasmid DNA, 1 µg of wild-type or mutant GFP or mCherry-TRα1 expression plasmid were transfected into cells either alone (GFP or mCherry) or together (GFP and mCherry).

For hormone treatment, the same experimental procedure was followed as described. However, after transfection, the transfection medium was replaced with MEM containing 10% charcoal-stripped FBS (minus T_3) where 100 nM T_3 (plus T_3) could then be added. A 100 nM thyroid hormone concentration is the standard concentration used in most studies (Bondzi et al., 2011; Bunn et al., 2001; Grespin et al., 2008; Mavinakere et al., 2012; Nagl et al. 1995).

Fluorescence Microscopy Analyses

Since the late twentieth century, there has been significant progress in the methods used to analyze the complex biochemical processes within cells. Fluorescence microscopy is a powerful method used to analyze the subcellular localization, transport routes, and binding interactions of fluorescent proteins in living cells (Lippincott-Schwartz et al., 2001). Cells transfected with expression plasmids for fluorescent proteins, such as GFP and its variants, contain highly accurate information about the spatial organization of the target proteins they are bound to, allowing for analysis of their movement within cellular compartments (Lippincott-Schwartz et al., 2003; Betzig et al., 2006). Therefore, the movement patterns of fluorescently tagged wild-type or mutant TRα1 was analyzed using fluorescence microscopy.

Semi-quantitative analysis

The semi-quantitative method of analysis relies on the categorization of transfected cells into three distinct categories based on the distribution of TRα1 within the cell (FIG.6). If TRα1 is primarily distributed to the nucleus, the cell is classified as having a nuclear TRα1 localization. In a similar fashion, if TRα1 has a distinct cytosolic population but still is distributed mostly to the nucleus then the cell is classified as having a combined nuclear and cytoplasmic localization. Finally, if TRα1 is distributed throughout the nucleus and cytoplasm without a clear distinction between the two compartments, the cell is classified as having a whole cell localization. For this method, a sample of at least two hundred cells was counted, each cell counted was sorted into its representative category, and the percent of each category was taken. Bar graphs were made and statistical analyses were performed using Microsoft Excel 2013.

Quantitative analysis

A quantitative method of fluorescence microscopy analysis was developed during this thesis research. The quantitative method is based on the utilization of region of interest (ROI) squares to compare the fluorescence intensity between the nucleus and cytoplasm of the cell (see Appendix for details).

Cell scoring

A semi-quantitative method of analysis was initially used until a quantitative method of analysis was developed. For both analyses, an inverted Nikon ECLIPSE TE

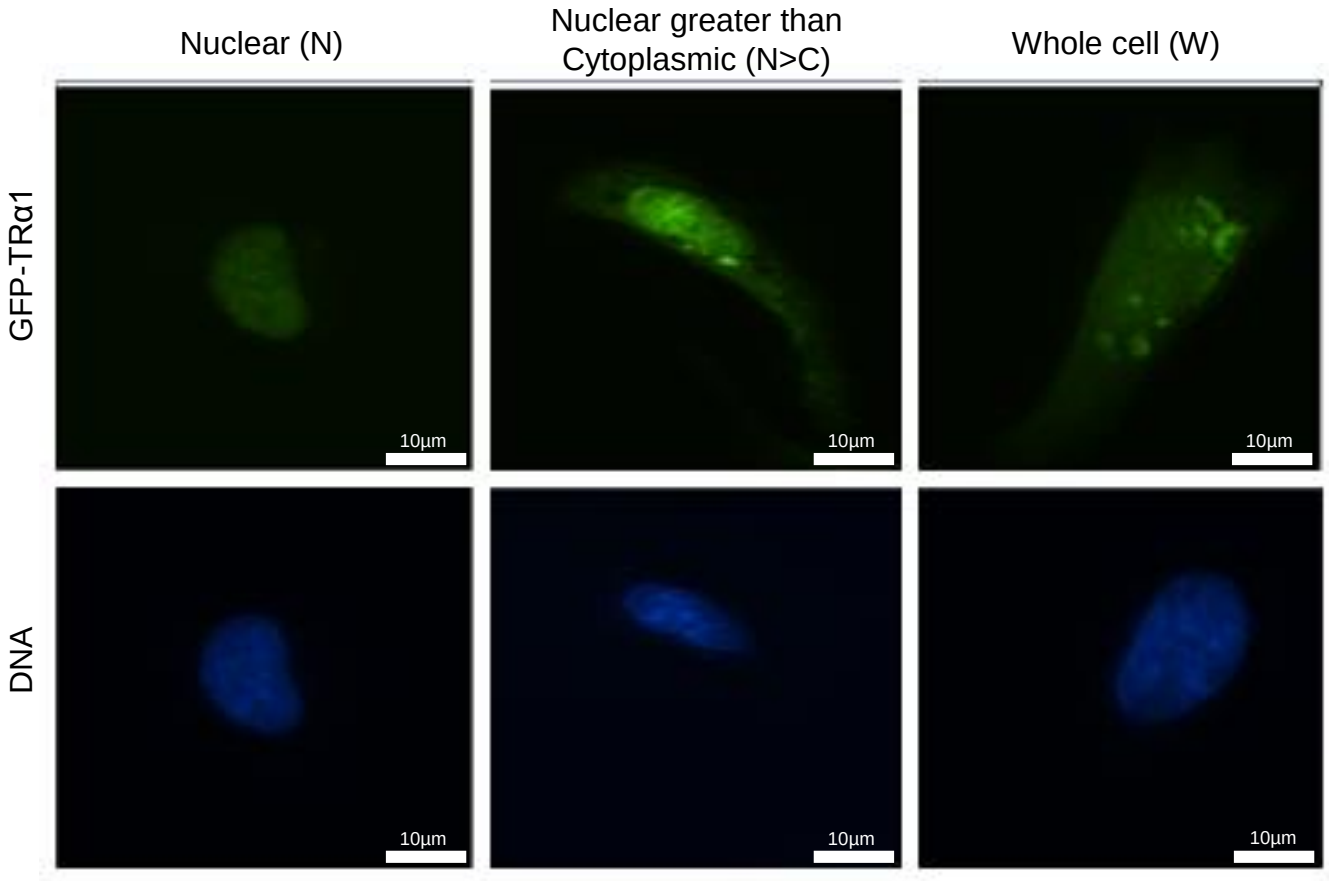


Figure 6. Semi-quantitative scoring categories
HeLa cells transfected with GFP-TRα1. Cells were scored as nuclear, nuclear plus cytoplasm, and whole cell. These categories were used to score the GFP or mCherry TRα1 nonacetylation mimic. In normal conditions GFP-TRα1 predominantly localizes to the nucleus.

2000-E fluorescence microscope (Nikon Ultraviolet Excitation: UV-2E/C filter block for DAPI visualization; Blue Excitation: B-2E/C filter block for GFP visualization; Red Excitation: T-2E/C filter block for mCherry visualization) was used with a Nikon Plan Apo 40x/0.75 objective. A CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ) and NIS-Elements AR software was used for image acquisition and primary image processing.

For the semi-quantitative method of analysis, the localization of wild-type or mutant GFP or mCherry-TRα1 was scored in either one of three categories: nuclear, nuclear and cytoplasmic, or whole cell. Data were quantified as the percentage of cells in a given category.

For the quantitative method of analysis, region of interest (ROI) squares were used to compare relative fluorescence intensity between the nucleus and cytoplasm to determine average nuclear/cytoplasmic (N/C) ratio.

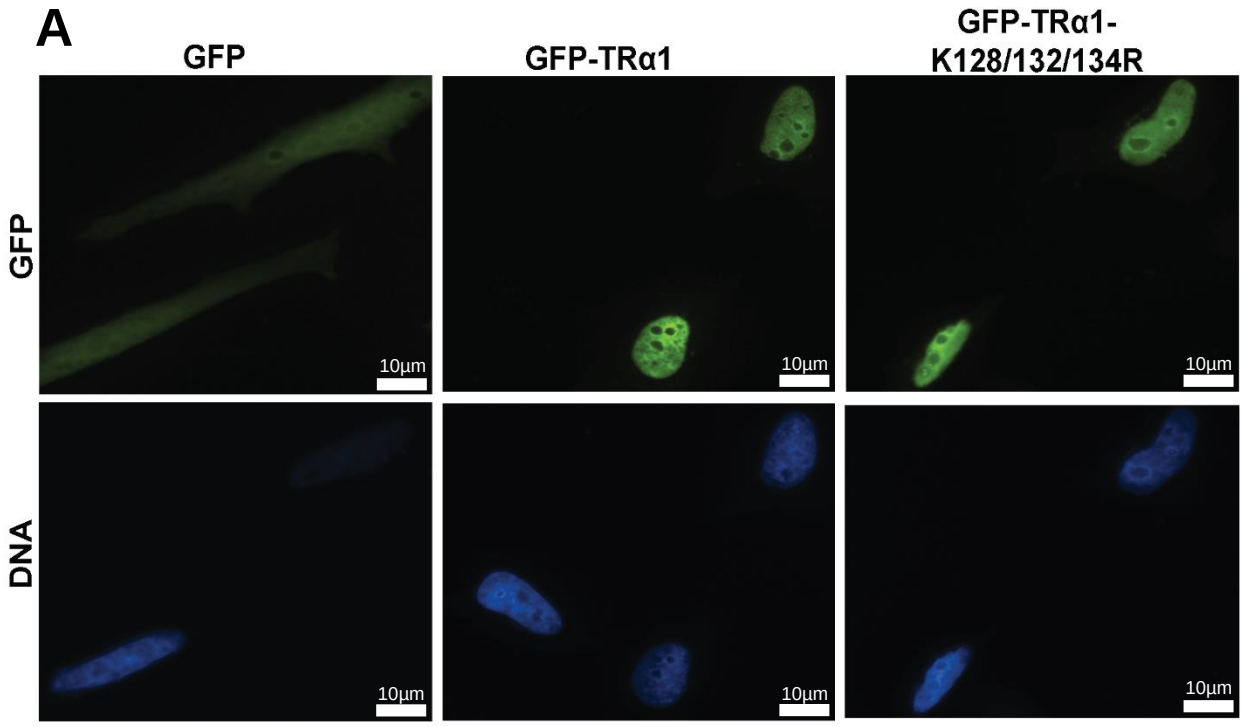
For both methods, cells were scored blind without knowledge of the treatment conditions. The slides' original labels were removed and replaced with random number or letter labels by another lab member, who made a key and kept it secure until the scoring was completed and data were analyzed. All experiments consisted of a minimum of 3 replicates and at least 100 cells were scored per replicate.

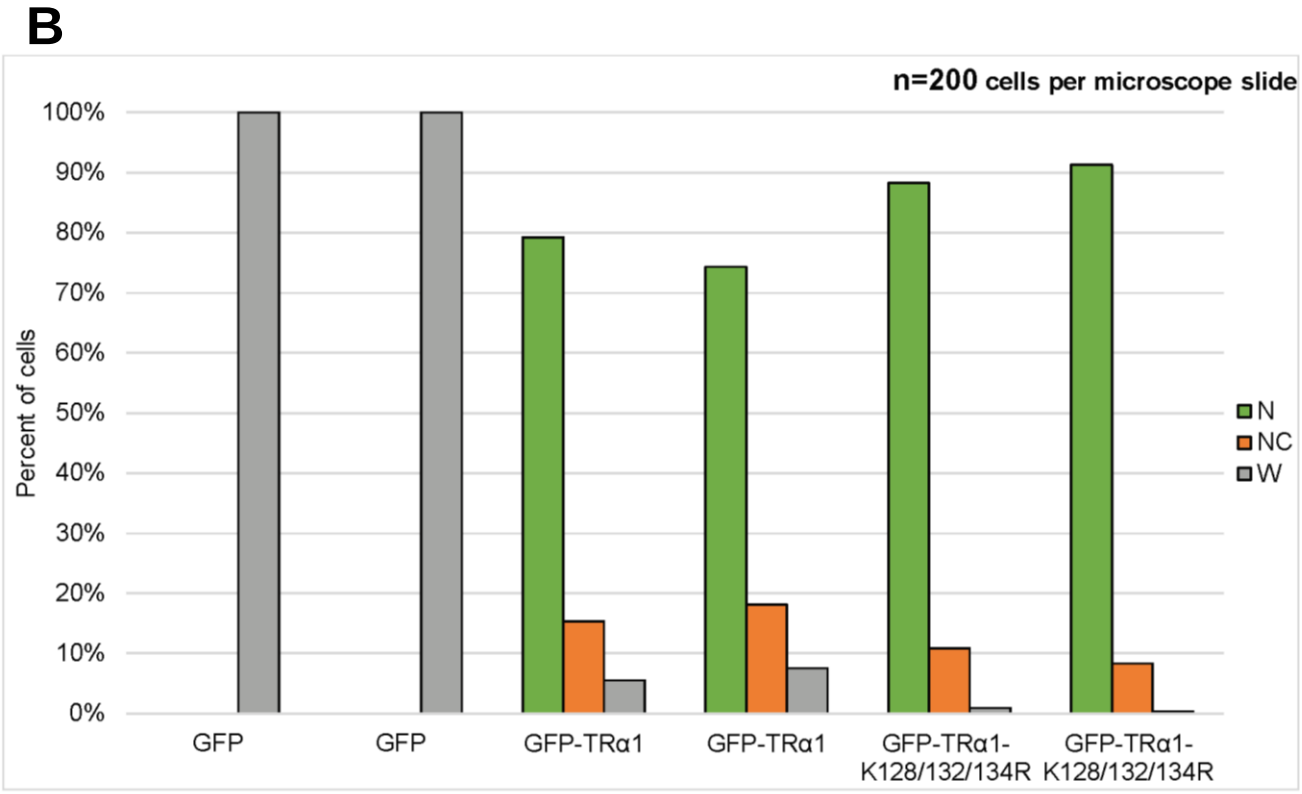
To minimize any factors that could have affected the accuracy of the data, care was taken be consistent in the timing of scoring of slides to avoid the gradual loss of fluorescence intensity over time, in the length of time cells were exposed to microscope fluorescent light, and in the placement of ROI squares within the nucleus or the cytoplasm of the cell.

Results

TRα1 nonacetylation mimic and wild-type TRα1 have similar nuclear localization patterns

Both GFP-tagged and mCherry-tagged TRα1 nonacetylation mimic were constructed to ensure that there were no tag-specific effects on distribution. HeLa cells transfected with GFP-TRα1 nonacetylation mimic had a primarily nuclear localization of TRα1, comparable to cells transfected with wild-type GFP-TRα1 (FIG.7). Likewise, similar results were seen with the mCherry-TRα1 nonacetylation mimic and mCherry-TRα1. The results show that at steady state 70% to 90% of transfected cells have a primarily nuclear localization. For both graphs, the y-axis represents the percent of cells and the x-axis represents the subcellular distribution of TRα1. Scoring categories were nuclear (N), nuclear plus cytoplasmic (NC), and whole cell (W), and a sample size of 200 cells was counted per microscope slide. These preliminary data consisted of multiple slides made in one transfection experiment (technical replicates), with 200 cells scored per slide. For GFP, GFP-TRα1, and GFP-TRα1 nonacetylation mimic there was one technical replicate each, and for the mCherry-TRα1 nonacetylation mimic there were three technical replicates. The data show that the TRα1 nonacetylation mimic shares a similar localization pattern to wild-type TRα1 at steady state (FIG.8). Similar trends seen in both GFP and mCherry provide validation for this result. A replicate experiment of the TRα1 nonacetylation mimic was used to compare the quantitative method of analysis with the semi-quantitative method analysis. Both methods of analysis yielded comparable results.





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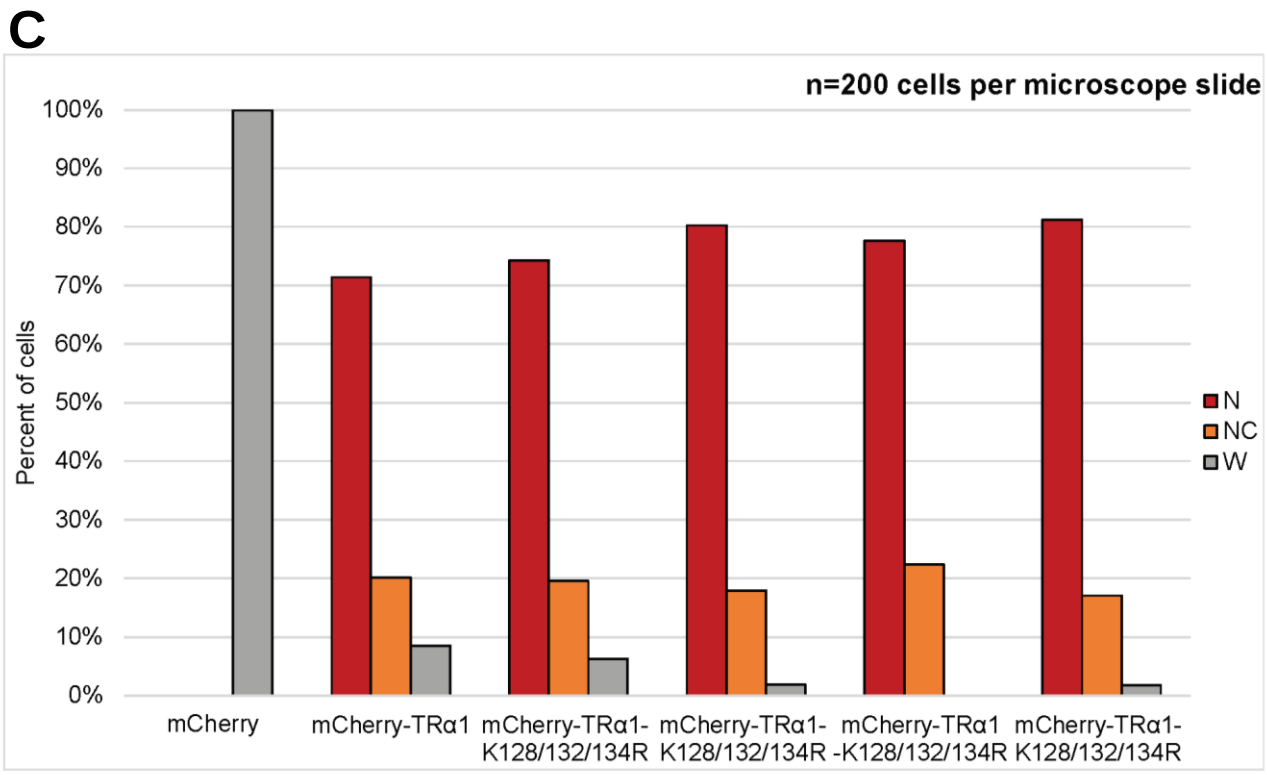


Figure 7. TRα1 nonacetylation mimic and wild-type TRα1 have similar nuclear localization patterns

Intracellular distribution pattern of TRα1 nonacetylated mimic (lysine to arginine substitutions) compared to wild-type TRα1. A) HeLa cells transfected with GFP, GFP-TRα1, and GFP-TRα1-K123/132/134R (and mCherry analogs) expression plasmids were fixed and analyzed by fluorescence microscopy using semi-quantitative scoring, after staining with DAPI to visualize DNA. Bars indicate B) one technical replicate of GFP, GFP-TRα1, GFP-TRα1-K123/132/134, and C) three technical replicates for mCherry-TRα1-K123/132/134R.

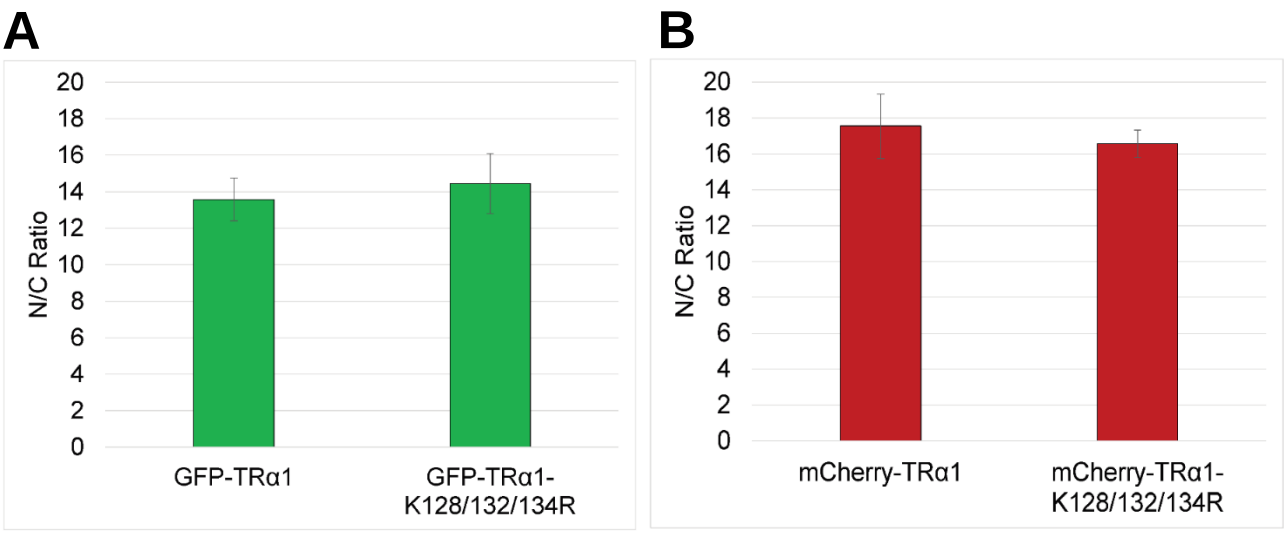


Figure 8. TRα1 nonacetylation mimic and wild-type TRα1 have similar nuclear localization patterns (2)

Distribution pattern of a nonacetylated TRα1 mimic (lysine to arginine substitutions) compared to wild-type TRα1. HeLa cells transfected with GFP-TRα1, and GFP-TRα1-K123/132/134R or mCherry analogs expression plasmids were fixed and quantitatively analyzed for TRα1 localization. For both A) GFP and B) mCherry graphs, the y-axis represents the N/C ratio, the x-axis represents the proteins analyzed. Bars indicate the nuclear/cytosolic ratio of TRα1 (n=3 independent, biologically separate replicate experiments, with 100 cells per replicate), and error bars indicate plus or minus standard error of the mean. P=0.640 > 0.05; student's t-test.

Thyroid hormone does not affect the localization of either TRα1 nonacetylation mimic or wild-type TRα1

The next step was to determine whether thyroid hormone has a ligand dependent effect on either wild-type TRα1 or TRα1 nonacetylation mimic transport activity. The data obtained from semi-quantitative analysis of subcellular distribution suggest that thyroid hormone has no effect on the transport activity of either the wild-type or TRα1 nonacetylation mimic (FIG.9).

Co-transfection of TRα1 nonacetylation mimic with wild-type TRα1 has no effect on wild-type TRα1 localization pattern

The research conducted by Sanchez-Pacheco et al. (2009) revealed that not only did the TRα1 nonacetylation mimic lose its ability to activate transcription at high thyroid hormone concentrations, it also acted as an inhibitor to wild-type TRα1's transcriptional activity. To determine whether this inhibition carried over to TRα1's transport activity, mCherry-wild-type TRα1 was co-transfected with GFP-TRα1 nonacetylation mimic or GFP-wild-type TRα1 with mCherry-TRα1 nonacetylation mimic. The nonacetylation mimic did not show an inhibitory effect on TRα1 (FIG.10).

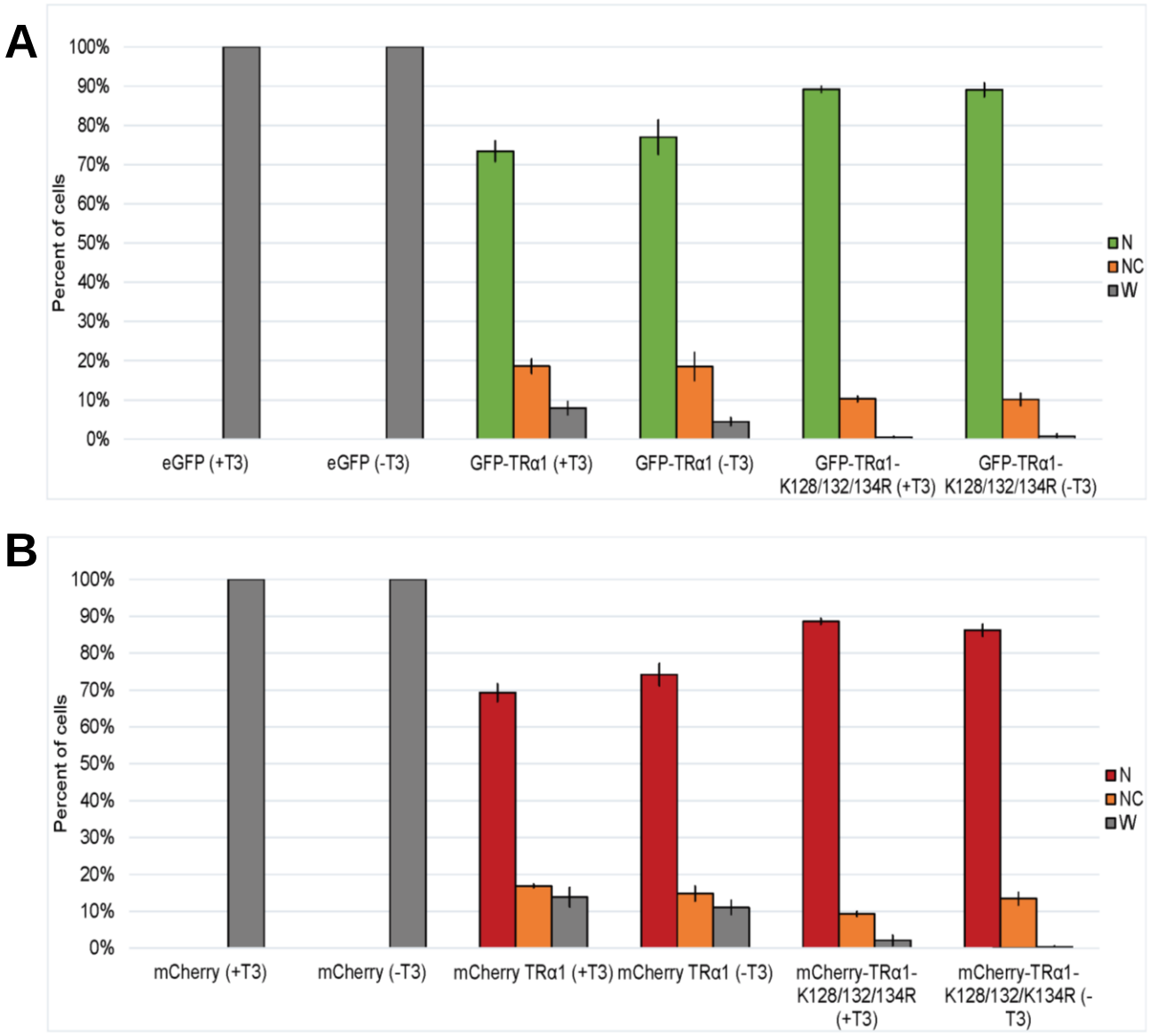


Figure 9. Thyroid hormone does not affect the localization of either TRα1 nonacetylation mimic or wild-type TRα1

HeLa cells were transfected with GFP, GFP-TRα1, and GFP-TRα1-K123/132/134R (A) or mCherry analogs (B) expression plasmids, and thyroid hormone treatment of 100nM was added 6 hours post transfection. Cells were sorted into N, NC, or W categories. The y-axis for both graphs represent the percent of cells with either N, NC, or W localization patterns, and the x-axis represents the proteins analyzed along with plus or minus thyroid hormone. Two-hundred cells were counted per slide, and at least three biologically separate replicates were analyzed. Error bars indicate plus/minus the standard error of the mean, and a student's t-test with a significance value of P=0.268 > 0.05 provides statistical evidence that thyroid hormone does not affect localization. Both wild-type and TRα1 nonacetylation mimic retain high nuclear localization.

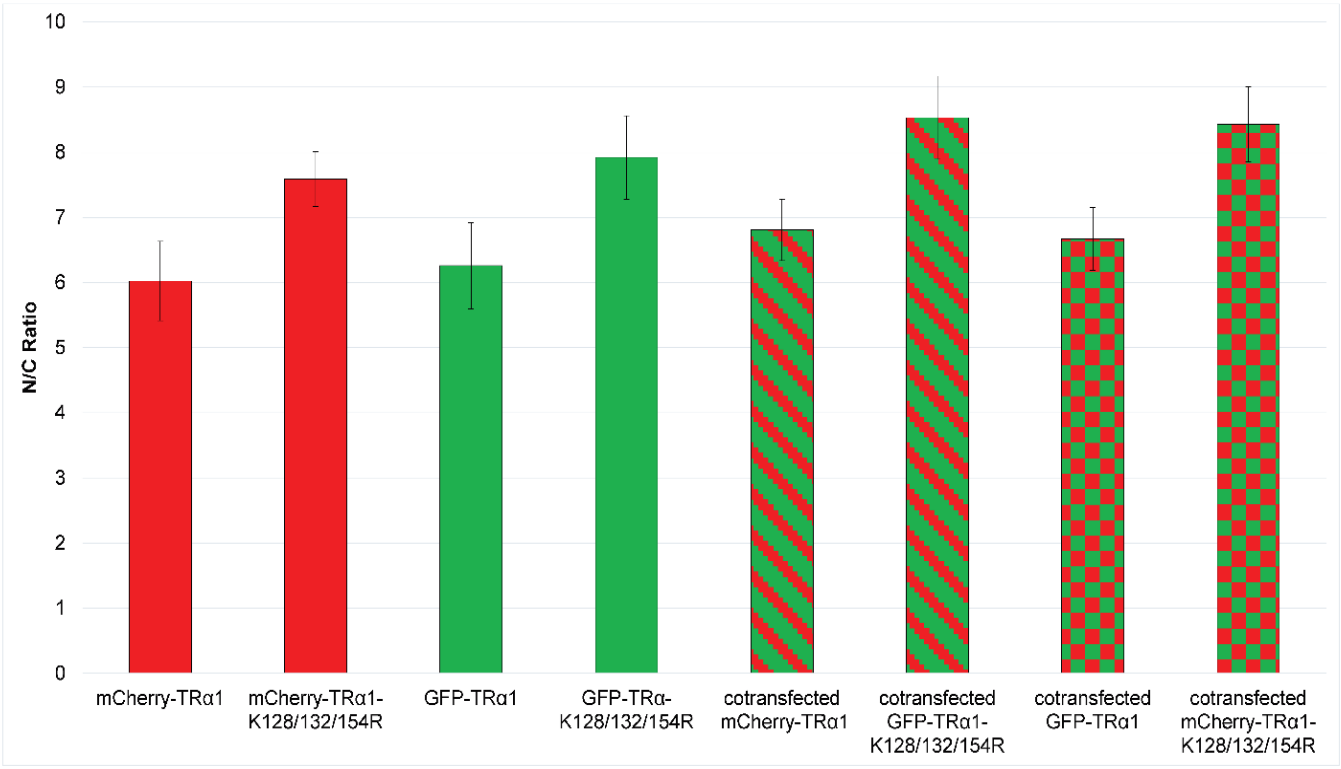


Figure 10. Co-transfection of TRα1 nonacetylation mimic with wild-type TRα1 has no effect on wild-type TRα1 localization pattern

HeLa cells were transfected in a six-well plate with GFP-TRα1 and GFP-TRα1-K123/132/134R (green), or mCherry-TRα1 and mCherry-TRα1-K123/132/134R (red), and the N/C ratio quantified by fluorescence microscopy. Striped bars represent one well of cells transfected with both mCherry-TRα1 and GFP-TRα1-K123/132/134R. Checkered bars represent one well of cells transfected with both GFP-TRα1 and mCherry-TRα1-K123/132/134R. Error bars indicate plus or minus standard error of the mean. P=0.767 > 0.05; student's t-test.

TRα1 acetylation mimic shows a reduced nuclear localization compared to wild-type TRα

In the previous analysis of the TRα1 nonacetylation mimic, the data showed that nuclear localization remains the same as wild-type TRα1, and that transport activity of the TRα1 nonacetylation mimic does not interfere with wild-type TRα1's transport activity. Thus, the next step was to determine the localization of the TRα1 acetylation mimic. Preparation of the GFP-acetylation mimic was unsuccessful via subcloning; therefore, HeLa cells were transfected with only mCherry-TRα1 acetylation mimic. The data showed a statistically significant decrease in nuclear localization for the GFP-TRα1 acetylation mimic compared to wild-type TRα1 (FIG.11).

Thyroid hormone does not affect localization of the TRα1 acetylation mimic

With the more cytosolic localization pattern of the TRα1 acetylation mimic established, the next step was to determine whether thyroid hormone would alter its distribution Consistent with the previous experiment on the effect of thyroid hormone on the localization of the TRα1 nonacetylation mimic, thyroid hormone had no effect on the localization of the TRα1 acetylation mimic (FIG.12).

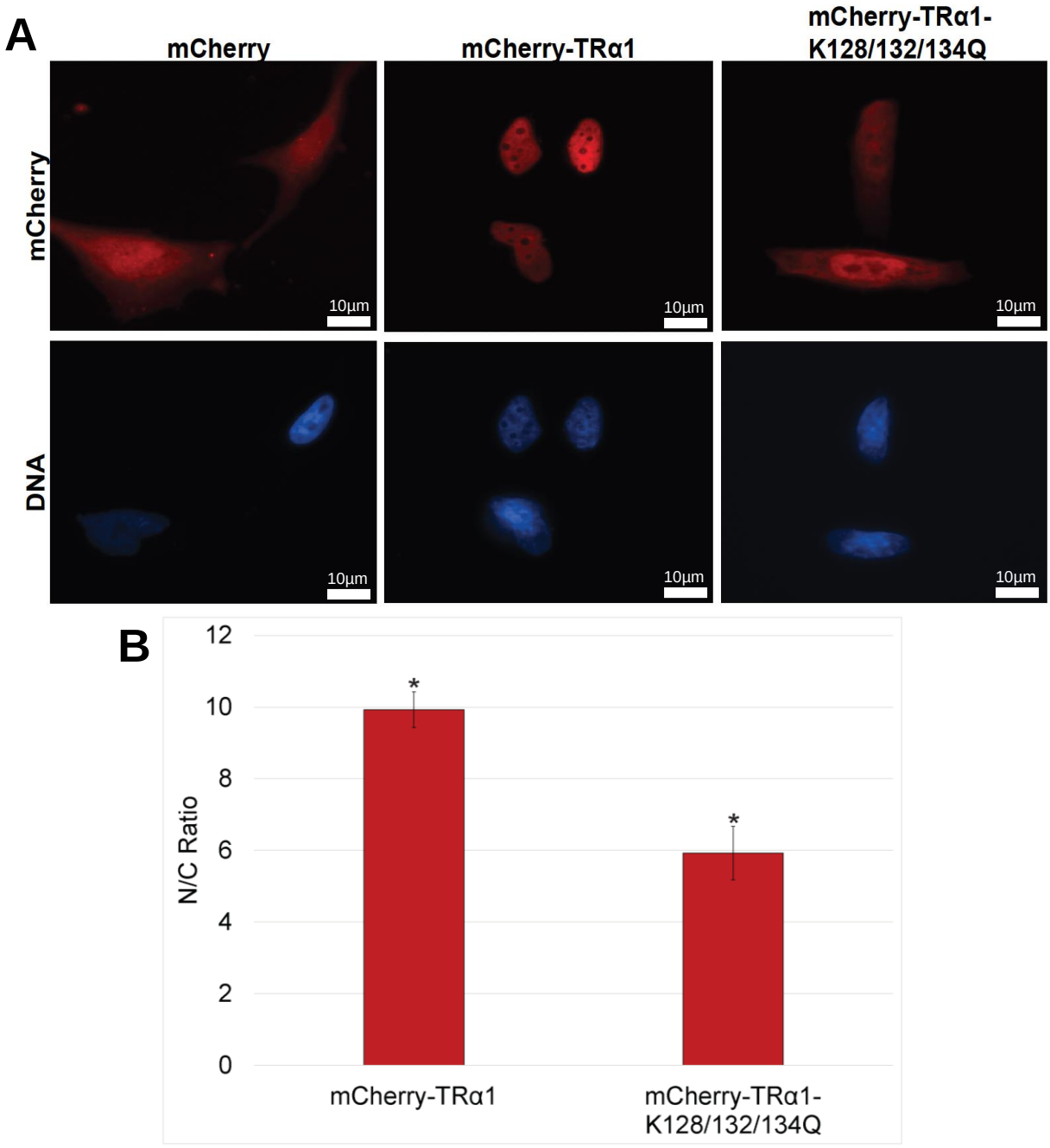


Figure 11. TRα1 acetylation mimic shows a reduced nuclear localization compared to wild-type TRα

Intracellular distribution pattern of the TRα1 acetylation mimic (lysine to glutamine substitutions) compared to wild-type TRα1. A) HeLa cells were transfected with mCherry, mCherry-TRα1, and mCherry-TRα1-K128/132/134Q expression plasmids, and analyzed by quantitative fluorescence microscopy. B) The y-axis represents the N/C ratio; the x-axis represents the proteins analyzed. Bars indicate the nuclear/cytosolic ratio of TRα1 (n=3 independent, biologically separate replicate experiments, with 100 cells per replicate), and error bars indicate plus or minus standard error of the mean. *P=0.011 < 0.05; student's t-test.

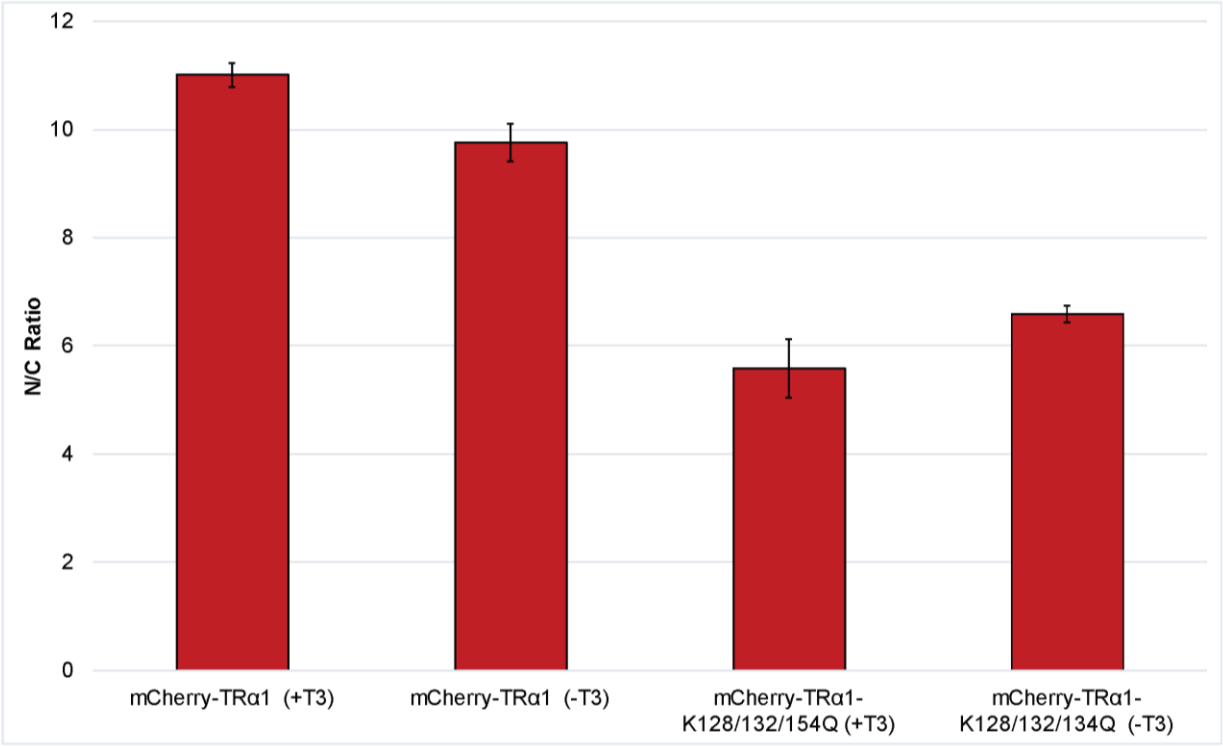


Figure 12. Thyroid hormone does not affect localization of the TRα1 acetylation mimic

Cells were transfected and treated with 100nM thyroid hormone as described in FIG.9. Bars indicate the nuclear/cytosolic ratio of TRα1 (n=3 independent, biologically separate replicate experiments, with 100 cells per replicate), and error bars indicate plus and minus the standard error of the mean. *P*=0.150 > 0.05; student's t-test.

Discussion

The data presented in this thesis show that the TRα1 nonacetylation mimic (lysine to arginine substitutions) is localized primarily to the nucleus, comparable to wild-type TRα1. Consistent with this finding, co-transfection of the TRα1 nonacetylation mimic did not alter the localization pattern of wild-type TRα1.

In striking contrast, the distribution of the TRα1 acetylation mimic (lysine to glutamine substitutions) was shifted towards a more cytosolic localization. Lastly, localization patterns remained the same in the presence or absence of thyroid hormone for wild-type or TRα1 nonacetylation and acetylation mimics.

Acetylation plays a role in TRα1 transport

The results presented here point to the possibility that acetylation may play an important role in TRα1's subcellular localization. Because acetylation occurs within the hinge domain NLS-1, it is highly likely that acetylation may function to regulate TRα1 import, by decreasing the affinity of NLS-1 for interaction with importins. Similar research conducted using different proteins support the finding that acetylation may modulate nuclear import. Song et al. (2015) found that the subcellular localization of Rho GTPase Net1A is controlled by acetylation within the NLS of this protein. To determine the effect of Net1A acetylation, acetylation sites in the protein were mutated into acetylation-mimic glutamine residues and nonacetylation-mimic arginine residues. While the nonacetylation-mimic did not have a change in nuclear localization, the acetylation-mimic showed a significant cytosolic localization. Prior to this work, it had been found that acetylation markedly alters the subcellular cellular location of the viral

oncoprotein E1A (Madison et al., 2002); non-receptor tyrosine kinase c-Abl (Bari et al., 2006); DNA helicase RECQL4 (Dietschy et al., 2009); immune response protein IFI16 (Li et al., 2012); and the S-phase kinase associated protein 2 (Inuzuka et al., 2012). Acetylation of these proteins was investigated with the use of acetylation mimics, and in all cases acetylation in the NLS promoted cytosolic localization. This thesis research contributes to the existing knowledge of the importance of acetylation in regulating NLS activity.

Electrostatic charge interactions may influence importin binding

This thesis research also provides evidence for an elegant model for the mechanism of interaction that takes place between importins and TRα1. An explanation as to why the TRα1 acetylation mimic had a distinct shift towards a more cytosolic localization may be due to electrostatic charge interactions between the NLS and importins. This form of interaction has been recently proposed in work conducted with the androgen receptor (Zhou et al., 2010). Zhou et al. identified a novel NES from the rice field eel androgen receptor containing a negative charge, and posited that the negative charge found in the NES may be indicative of an export pathway mediated by electrostatic interactions. In addition, structural studies by Gino et al. (2002) revealed that interaction of importin β with its cargo occurs via electrostatic interactions, enabled by acidic amino acid side chains along its surface. Furthermore, research on the nuclear export factor CRM1 reveals a method for modulating its binding with NESs. CRM1 changes its structure to expose or bury acidic or basic amino acid residues that lie on either the outer surface or inner surface of the protein (Fox et al. 2011). Theoretical

work by Zhang et al. (2011) suggests that protein-protein electrostatic interactions begin with the formation of transient intermediates that then relax into stable complexes. Thus, taking into account the physiological pH of HeLa cells and the fact that importins of the β family are negatively charged, it is probable that positively charged lysine residues 128,132,134 on TRα1 are neutralized due to acetylation. As a consequence, the binding affinity of NLS-1 for importin 7, importin β1, and the adaptor importin α1 complex (Roggero et al., 2016) is likely to be significantly reduced.

Support for the regulatory activity of acetylation

Not only does acetylation play a role in the subcellular localization of NLS-containing proteins, it can also regulate processes that play a part in cytoskeleton remodeling, cell migration, metabolism, and aging (Close et al., 2010). Zhao et al. (2010) showed that acetylation occurs in almost every enzyme that catalyzes cellular metabolism as a result to changes in extracellular nutrient availability. In addition, Nguyen et al. (2016) discovered that acetylation regulates the activity of the enzyme glutamine synthetase. Other roles for this post-translational modification include acetylation of histone H3 lysine 23, which regulates gene expression responsible for the development of *Drosophila melanogaster* (Bodai et al., 2012); acetylation of transcription factors responsible for the differentiation and maintenance of quiescence in adult hematopoietic stem cells (Bararia et al., 2016); and regulation of the essential functions of the small GTP-binding protein Ran (De Boor et al., 2015; Knyphausen et al., 2015). Gorsky et al. (2016) constructed a lysine to glutamine acetylation mimic, and a lysine to arginine nonacetylation mimic of the human protein Tau (hTau) to observe

the contribution of acetylation to hTau toxicity, as observed in many neurodegenerative disorders such as Alzheimer's disease (AD). They found that the acetylation of the single K128 residue in hTau is enough to worsen hTau neurotoxicity *in vivo*, suggesting that acetylation of hTau contributes to the events leading to neurodegeneration in AD.

Conclusion

Prior studies show that TRα1 rapidly shuttles between the cytoplasm and nucleus of the cell (Bunn et al., 2001). Multiple exportins mediate its export pathway (Grespin et al., 2008; Subramanian et al., 2015), and import of TRα1 into the nucleus is mediated via a number of importins, specifically importin 7, importin β1, and the adaptor importin α1 (Roggero et al., 2016). However, the exact mechanism of interaction between transport factors and TRα1 remains unclear. This thesis work proposes a mechanism of electrostatic charge interactions in which transport factors may interact with TRα1 to facilitate nucleocytoplasmic translocation. Even though the proposed mechanism might be a possible explanation as to the functional consequence of TRα1 acetylation, it is still unknown as to why the receptor is initially acetylated. Is acetylation a regulatory checkpoint in TRα1 transport? What are the signals that result in nuclear receptor acetylation? What is the evolutionary significance of TRα1 acetylation? These questions, and many more, still remain to be answered in order to fully understand the complete story behind TRα1 acetylation.

Future Directions

Future steps include conducting co-transfection experiments to determine the effect of the TRα1 acetylation mimic on wild-type TRα1 localization. In addition, work is currently done by a graduate student in our lab, Dylan (Jibo) Zhang, to verify that the TRα1 nonacetylation mimic is, indeed, nonacetylated, by GFP/RFP-trap co-immunoprecipitation using an acetylated lysine-specific antibody. Further mutagenesis experiments in which TRα1's NLS-2 in the A/B domain is removed will be done to determine whether NLS-2 accounts for the fact that the acetylation mimic can still localize, in part, to the nucleus. Finally, work with TRβ will be done to determine the functional consequences of acetylation on TRβ's subcellular localization.

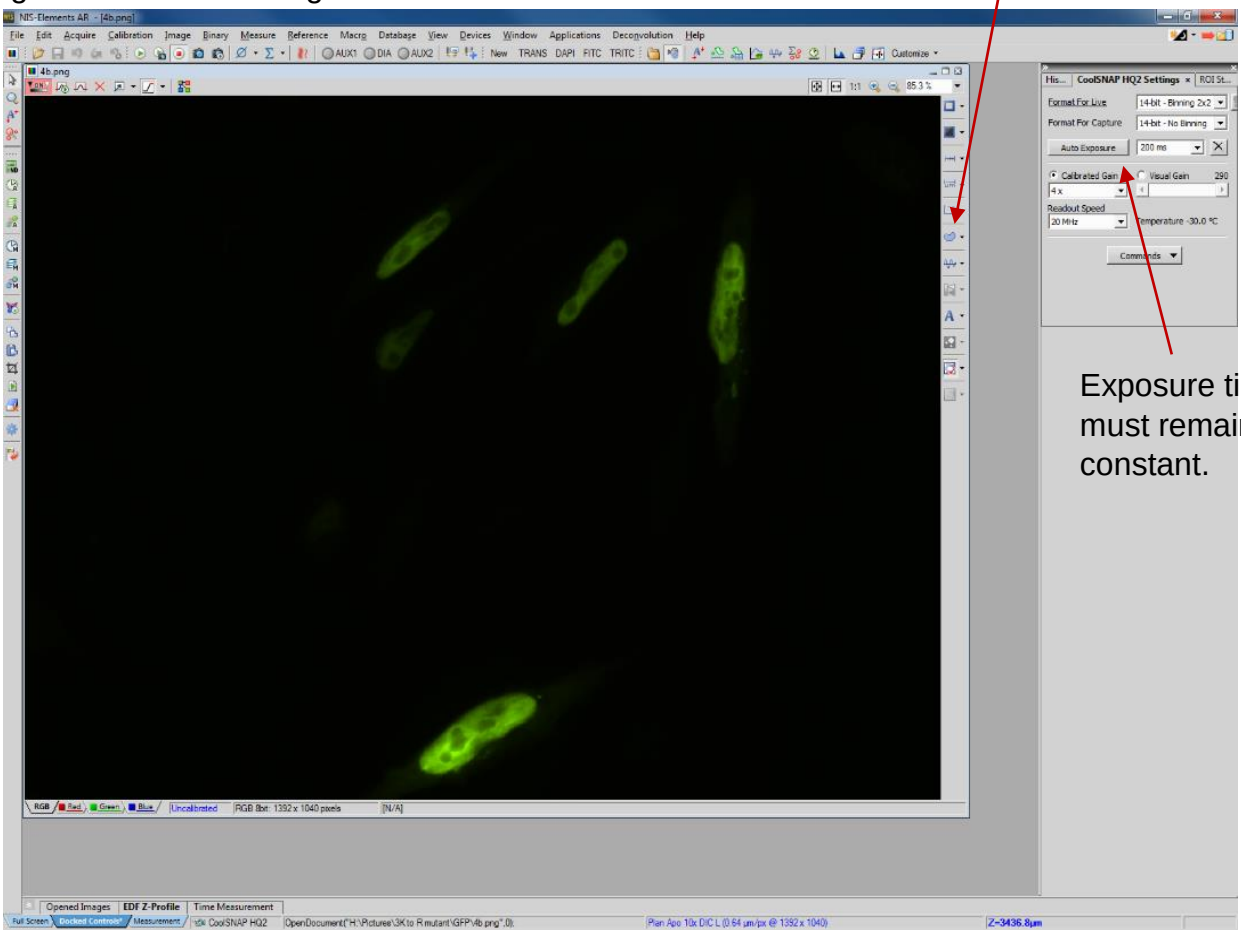
Appendix

Detailed protocol for Quantitative Microscopy Analysis

Program: NIS-Elements

Access to ROIs:

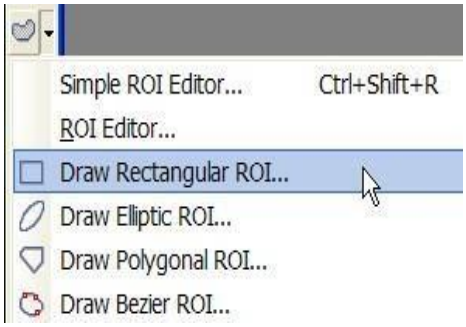
To create ROIs on an image, click the small black arrow next to the ROI icon on the right border of the image window.



(Cells transfected with GFP-TRα1-K128/132/154R)

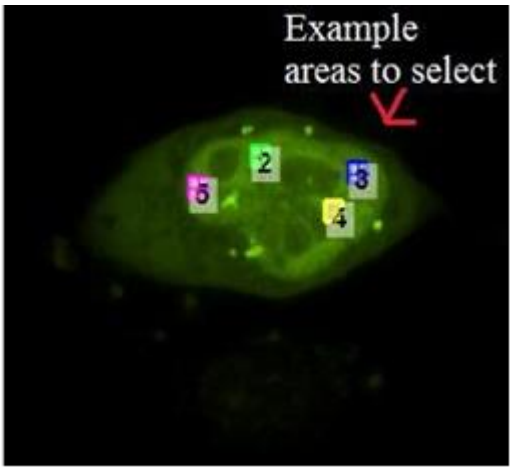
Options for creating ROIs:

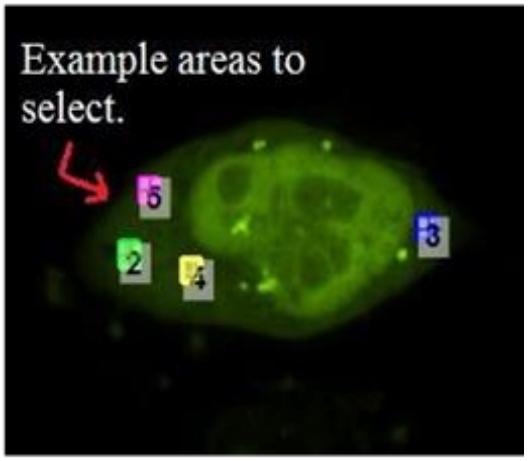
Draw ROI using the Draw Rectangular ROI:



Drawing the ROIs on the image:

- Select the ROI form (for this example Draw Rectangular ROI was chosen).
- Draw around selected area.
- When done, right click to duplicate ROI.
- Another ROI with similar dimensions will appear.



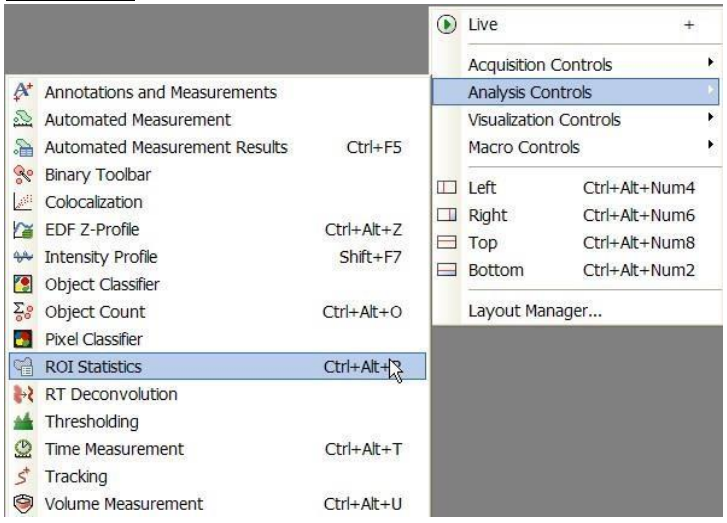


(Cell transfected with GFP-TRα1)

These are sample sizes and areas to select. Only select smooth, homogenous areas of GFP-TRα1 in the cell. Labeling of ROI's can be arbitrary as long as a system to distinguish between the nucleus and cytoplasm is developed.

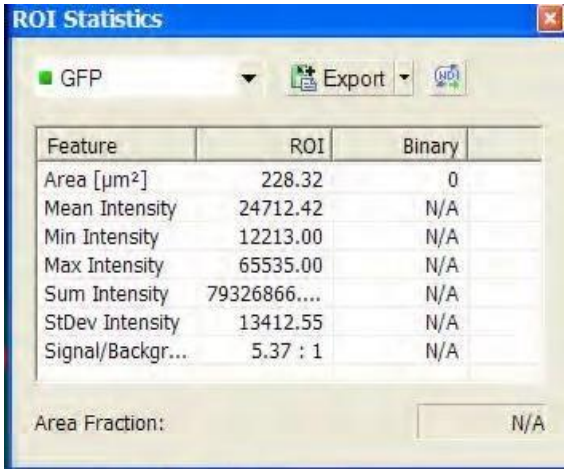
Access to ROI Statistics:

Right click on the NIS-Elements desktop and select Analysis Controls, then select ROI Statistics.



Using ROI Statistics Dialog:

- ROI statistics will report Area, Mean, Min, Max, Sum StDev Intensity and the ratio of Signal/ Background.
- What you are interested in is the Mean Intensity.



Exporting Data from ROI Statistics:

To export ROI data:

- Select the small black arrow to view a drop down of possible export locations.
- Select the desired export location.

- Click on the Export button.

- Once exported save the Excel file to the name of your microscope slide.
- After scoring, copy and paste ROI ID and ROI Mean unto a new excel sheet.
- Sort ROI Mean such that the data from the nucleus is on top of the data from the cytoplasm.
- Divide each cells nuclear (N) over cytoplasmic data (C) to determine the N/C ratio.
- Take the average of each cells N/C ratio, then use that average to create a bar graph.

Adapted from Nikon Instruments Inc., 2013

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