

**UNDERSTANDING THE ROLE OF THE HINGE
DOMAIN IN INTERDOMAIN COMMUNICATION IN
NUCLEAR HORMONE RECEPTORS**

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DEDICATION

To God Almighty and the Dompkeh family.

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ABSTRACT

Nuclear hormone receptors (NRs) are eukaryotic transcription factors that modulate the expression of target genes to regulate physiological processes such as metabolism, reproduction, and development. NRs are modular proteins with distinct domains whose activity is modulated by interactions with lipophilic small molecule ligands, DNA, and other co-regulators. NR interactions with these molecules induce structural changes that sometimes allow interdomain communication between the DNA-binding domain and the ligand-binding domain. This communication between the structured domains is increasingly recognized as a regulatory mechanism among NRs. An intrinsically disordered peptide hinge region links the more structured DNA-binding (DBD) and ligand-binding domains (LBD). Studies have reported distinct extended or ‘open’ and ‘closed’ conformations of these nuclear receptors. These conformations have been associated with the ligated state of the NR. Here, we studied the structural changes in the ligand-bound state of the thyroid hormone receptor (TR) in the presence and absence of DNA using single-molecule Förster Resonance Energy Transfer (smFRET). We observed both open and closed states in DNA-bound and DNA-free TR. These observations, though preliminary, suggest that the structural changes that occur in TR are transient and dependent on DNA binding. However, rather than having one distinct conformation correlate to a DNA-bound state, it is more of a “tapping” motion that closes and releases in an unstable manner, with DNA binding increasing stability of the NR.

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CHAPTER ONE

INTRODUCTION

Nuclear hormone receptors (NRs) are ubiquitously expressed in all human tissue (Helsen & Claessens, 2014; Weikum et al., 2018) and are very essential to many physiological processes such as reproduction, metabolism, immunity, and cell signaling, among others (Mangelsdorf et al., 1995). They are implicated in many diseases (McKenna & O'Malley, 2010), including the androgen receptor (AR) in prostate cancer (Fujita & Nonomura, 2019), the glucocorticoid receptor (GR) in Cushing's disease (Lamberts, 2002), the estrogen receptor (ER) and progesterone receptor (PR) in breast cancer (Yip & Rhodes, 2014), and the thyroid receptor (TR) in thyroid disease (Vallortigara et al., 2009).

NRs are activated by small lipophilic molecules, which may act as agonists or antagonists (Burris et al., 2013; Moore et al., 2006). Ligand binding induces structural changes that enable dimerization, enhancing DNA binding in most nuclear receptors (Fischer & Smieško, 2019; Lavery & McEwan, 2005). Activated NRs bind to DNA hormone response elements (HREs) located in the regulatory regions of their target genes (Putcha & Fernandez, 2009). This allows NRs to interact with the transcription pre-initiation complex (PIC) (Lavery & McEwan, 2005) regulating transcription and subsequent gene expression (Jin & Li, 2010; Weikum et al., 2018) through coregulator recruitment (Kumar et al., 2013; Lavery & McEwan, 2005). With this, NRs function as molecular switches that integrate extracellular signals with regulated gene expression (Aagaard et al., 2011; McEwan, 2012).

Structurally, NRs are modular proteins composed of distinct domains: a variable N-terminal domain (NTD), a conserved DNA-binding domain (DBD), a flexible hinge region, and a ligand-binding domain (LBD) (Helsen & Claessens, 2014; Weikum et al., 2018). While the

structured domains, LBD and DBD, have been well characterized, increasing evidence highlights the importance of the N-terminal domain and the linker or “hinge” region that connects these LBD and DBD. Notably, these regions lack a defined tertiary structure and exhibit hallmarks of intrinsically disordered regions (IDRs) (Chakrabarti & Chakravarty, 2022; Helsen & Claessens, 2014; McEwan, 2012). Rather than serving as passive connectors, these flexible linkers are increasingly recognized as key determinants of NR behavior, facilitating structural adaptability, mediating allosteric communication, and influencing nuclear localization and interactions with coregulators (De Bosscher et al., 2020; Jin & Li, 2010; McEwan, 2012). These further expand the functional range of nuclear receptors beyond traditional models of receptor activity.

Allostery in NRs is increasingly understood as a regulatory mechanism (Fernandez, 2018). Allosteric modulation facilitates structural changes induced upon ligand or DNA binding (Changeux & Christopoulos, 2016). This dynamic nature of NRs has been revealed using high-resolution biophysical techniques such as crystallography (Putcha et al., 2012) and cryo-electron microscopy (cryo-EM) (Maletta et al., 2014). However, these techniques are limited in their ability to elucidate IDRs, which are essential for allosteric communication and functional diversity (Zanotti, 2023). IDRs often lack stable secondary or tertiary structures in isolation and are frequently omitted from structural models due to their intrinsic disorder and structural heterogeneity (Shamilov & Aneskievich, 2019; Zanotti, 2023). As a result, conventional structures provide only a partial view, failing to capture the full dynamic and allosteric behavior of these receptors (Chakrabarti & Chakravarty, 2022; McEwan, 2012).

Emerging biophysical techniques, including nuclear magnetic resonance (NMR) (Dyson & Wright, 2021), small-angle X-ray scattering (SAXS) (Dyson & Wright, 2021), and single-molecule Förster Resonance Energy Transfer (smFRET) (Agam et al., 2023), as well as

integrative modeling (Gomes et al., 2020) are used combinatorially with X-ray crystallography and cryo-EM to study the flexibility and conformational ensembles of NRs (Zanotti, 2023). These approaches hold promise for capturing the allosteric transitions and multidomain interactions that are central to receptor signaling but invisible in static crystallographic views (Seacrist et al., 2020).

Recent studies have reported distinct extended (or open) and closed conformations in nuclear receptors using this combinatorial approach (Chandra et al., 2017; Choi et al., 2023; Hazarika et al., 2024; Rastinejad, 2022, 2025). The presence of the disordered hinge domain allows for dynamic movement of the nuclear receptor (Haelens et al., 2007). This facilitates domain-domain communication between the two structured domains, the LBD and the DBD (Hazarika et al., 2024). This interdomain signaling is important for allosteric regulation, functional specificity, and structural adaptability (Rastinejad, 2025; Yu et al., 2025; Zwart et al., 2010). Also, the hinge contains a nuclear localization signal, and sites for post-translational modifications (Miyamoto et al., 2001) and physiologically relevant mutations (Kumarasamy et al., 2015). Despite these findings, intricate details of how the DBD and LBD communicate through allostery remain unknown.

In this study, we demonstrate that both open and closed states are present in DNA-bound and DNA-free forms of the thyroid hormone receptor (TR). We study the conformational changes induced by DNA binding to ligand-bound TR using single-molecule Förster Resonance Energy Transfer (smFRET). TR α (with IUPHAR/BPS nomenclature NR1A1) (Alexander et al., 2019) is mostly expressed in the brain and is associated with the development of the nervous system. Triiodothyronine (T3) is the primary endogenous agonist for the thyroid hormone receptor (TR), binding with high affinity to regulate gene expression (Yen, 2001). Unlike other

NRs, TR α is constitutively localized within the nucleus, where it interacts with nucleosomal DNA (Wong et al., 1995). In this study, we use the avian *Gallus gallus* ortholog of TR α (c-erbA, NR1A1-A), denoted cTRA. This ortholog has a >90% amino acid identity with human TR α (Putcha & Fernandez, 2009; Sechman et al., 2009). We generate unnatural amino acid (UAA) point mutations for the simultaneous attachment of fluoroprobes for smFRET spectroscopy.

CHAPTER TWO

LITERATURE REVIEW

Nuclear Receptor Structure and Function

Nuclear hormone receptors (NRs) are a conserved class of transcription factors whose transcriptional activity is modulated by small lipophilic ligands in response to hormonal (or environmental) and metabolic stimuli (Weikum et al., 2018). These receptors play critical roles in development, homeostasis, and disease, functioning as molecular switches that integrate extracellular signals with regulated gene expression. Structurally, NRs are modular proteins composed of distinct domains: a variable N-terminal domain (NTD), a conserved DNA-binding domain (DBD), a flexible hinge region, and a ligand-binding domain (LBD) as shown in **figure 2.1**. While the DBD and LBD are well studied, the hinge domain remains structurally elusive but functionally critical. This review explores its role in facilitating communication between domains.

The LBD is a highly conserved NR domain consisting of 12 α -helices arranged in a characteristic antiparallel fold (Helsen & Claessens, 2014; Li et al., 2021). Helix H12 acts as a lid that swings into place over the ligand-binding pocket upon ligand binding (de Vink et al., 2022). In the absence of ligand, H12 is flexible or displaced. When an agonist binds, H12 snaps into place. This conformational change exposes the activation function 2 (AF-2) surface needed to recruit coactivators and trigger gene transcription (Khan et al., 2022). Beyond ligand recognition, the LBD also contributes to receptor dimerization in several nuclear receptors, such as vitamin D receptor (VDR), thyroid receptor (TR), peroxisome proliferator-activated receptor (PPAR), retinoid acid receptor (RAR), and retinoid X receptor (RXR) (Helsen & Claessens, 2014). This dimerization interface typically involves helices H9 and H10 and the loop between helices H7 and H8 (Beinstein et al., 2022). However, evidence suggests that androgen (AR)

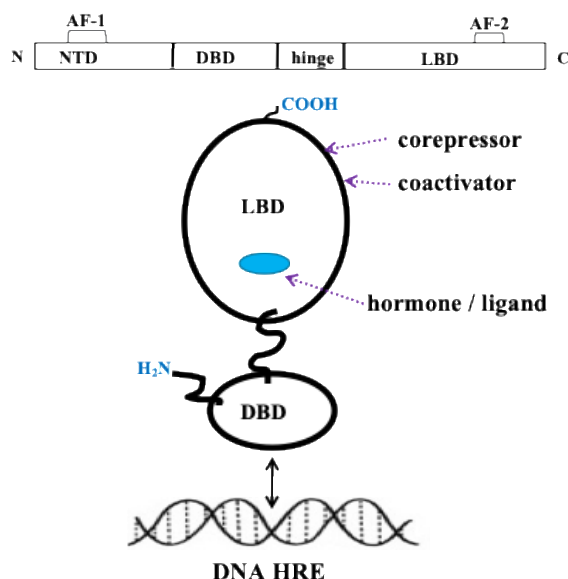


Figure 2.1. A schematic illustrating the general structure of nuclear hormone receptors. Hormone ligands and coregulators bind to the LBD, while the DBD interacts with DNA.

(Nadal et al., 2017), glucocorticoid (GR) (Lempiäinen, 2020), progesterone (PR) (Darbre, 2022), and mineralocorticoid receptors (MR) (Grossmann et al., 2022) do not dimerize through their LBDs (Klinge, 2018). Structural analyses show that these receptors crystallize as monomers, and the interactions observed in LBD crystal structures appear to result from crystal packing rather than biologically relevant contacts (McEwan, 2023). LBD structures, especially with agonist or antagonist bound, have been instrumental in guiding synthetic ligand development, computational screening, and understanding the functional consequences of nuclear receptor mutations (Helsen & Claessens, 2014). While AF-2 is the primary transactivation domain for most receptors, the NTD may also contain an activation function (AF-1) that contributes to gene regulation in receptors (Weikum et al., 2018).

The DBD is the most conserved region of NRs binding specific DNA sequences known as hormone response elements (HREs) (Helsen & Claessens, 2014). HREs are made of two

hexameric sequences, or half-sites, organized as direct, inverted, or everted repeats (Helsen & Claessens, 2014; Mangelsdorf et al., 1995). These half-sites are separated from each other by a spacer sequence with variable length. Each half-site is recognized by a receptor (Roemer et al., 2006). The selectivity of NRs binding to the HREs depends on the sequence composition of the half-sites and the length of the spacer. Receptors may bind as heterodimers with the retinoid X receptor (RXR), as homodimers, or as monomers (Helsen & Claessens, 2014; Mangelsdorf et al., 1995). Monomeric receptors are less common. Steroid receptors, such as the androgen receptor (AR), recognize the 5'-AGAACA-3'-like motifs, while non-steroid receptors and the estrogen receptor (ER) recognize the 5'-AGGTCA-3'-like motifs (Helsen & Claessens, 2014). For example, the thyroid receptor (TR), a non-steroid receptor can bind to the 5'-AGGTCA-3' HRE sequence as a monomer (half-site), a homodimer (DR4, IP6, P0), and as a heterodimer with the RXR (DR4) (Helsen & Claessens, 2014; Rastinejad et al., 1995). The DBD contains two zinc finger motifs. The first zinc finger contains an α -helix responsible for recognizing and binding specific DNA sequences, while the second zinc finger supports dimerization, enabling the receptor to form homo- or heterodimers (Helsen et al., 2012). Additionally, the DBD includes a variable C-terminal extension (CTE), which can enhance DNA binding by interacting with the minor groove or contribute to dimer stabilization through protein-protein interactions (Jiang et al., 2024; Mohideen-Abdul et al., 2017). Effective dimerization of the DBD is coordinated with the structure of the hormone response element, which may vary in spacer length and hexamer orientation (Helsen & Claessens, 2014; Weikum et al., 2018). Specificity in DNA binding enhances the regulation of the recruitment of coregulators and RNA polymerase machinery, thereby regulating gene expression (Denayer et al., 2010; Mangelsdorf et al., 1995).

The DBD has been known historically as the main player in NR-DNA binding mechanisms (Helsen & Claessens, 2014; Mangelsdorf et al., 1995) . However, some studies show that certain sequences in the NTD (Sheikhhassani et al., 2022) and hinge domain (Steiner et al., 2022) of some NRs interact directly with DNA and enhance their transcriptional effects (Zhao et al., 2024).

Intrinsically Disordered Regions in NRs

The sequences of the NTD and hinge are less conserved within the NR superfamily, their structures are intrinsically disordered, and their functional and regulatory roles at the molecular level are poorly understood (Lavery & McEwan, 2005). Studies have shown that mutations within these domains have resulted in malfunction in transcription facilitated by NRs (Hadzic et al., 1995; Pawlak et al., 2012). Rather than serving as passive connectors, IDRs within NRs contribute to interdomain flexibility, allosteric communication, and post-translational regulation (Chakrabarti & Chakravarty, 2022; Rastinejad, 2025; Zanotti, 2023). In addition, IDRs are increasingly implicated in mediating phase separation, subnuclear localization, and protein–protein interaction networks, further enhancing their range of function beyond canonical receptor activity (Li et al., 2025; Uversky, 2024; Zhang et al., 2024).

Interdomain Communication and Allostery in NRs

Allostery is increasingly recognized among NRs as a common regulatory mechanism (Changeux & Christopoulos, 2016; Rastinejad, 2025). Allosteric structural changes within nuclear receptors have been revealed using biophysical tools such as X-ray crystallography (Putcha et al., 2012), hydrogen-deuterium exchange mass spectrometry (HDX MS) (Wright et al., 2011), fluorescence (Fernandez et al., 2017), and NMR spectroscopy (Kojetin et al., 2015). Binding of the ligand to the LBD induces a conformational change that exposes the AF-2 and

increases DBD affinity for DNA (Hazarika et al., 2024; Rastinejad, 2025). Studies have reported a ‘closed’ or ‘open’ conformation facilitated by the hinge domain that links the DBD and the LBD (Chandra et al., 2013; Rochel et al., 2011). Despite these findings, intricate details of how the DBD and LBD communicate through allostery remain unknown.

Interdomain signaling between the DBD and LBD is critical for nuclear receptors to integrate signals from ligands, DNA, and coregulators, enabling precise control of gene expression (Hazarika et al., 2024; Rastinejad, 2025). This allosteric crosstalk determines DNA affinity, ligand sensitivity, receptor conformation, and downstream transcriptional outcomes, providing a total view of the NR molecule: rather than isolated domains, the full-length receptor is an integrated allosteric unit, with interdomain interfaces for signal transduction. NRs function as allosteric proteins that integrate signals from ligands, DNA, and cofactors to modulate gene expression. This integration relies heavily on the capacity of the LBD and DBD to communicate across flexible, often disordered hinge domain. Interdomain communication allows the receptor to adopt conformational states that coordinate ligand sensing with DNA response element binding, ultimately tuning transcriptional outcomes (Choi et al., 2023; Rastinejad, 2025). Despite being a hallmark of NR function, the precise mechanisms underlying this communication remain poorly understood, particularly in the context of full-length receptors.

Functional Importance of Interdomain Crosstalk

Interdomain communication is essential for nuclear receptors to act as integrated signaling units. Ligand binding to the LBD can allosterically influence DNA binding affinity and sequence selectivity, while DNA binding can reciprocally alter the conformation and dynamics of the LBD (Fernandez, 2018). This bidirectional communication allows the receptor to respond not just to the presence of a ligand, but also to the chromatin context and associated coregulatory

proteins. For example, different DNA response elements have been shown to elicit distinct conformational states in GR and ER (Chandra et al., 2017), suggesting that gene regulation is not solely determined by ligand identity, but also by the dynamic state of the full-length protein (Hazarika et al., 2024; Weikum et al., 2018). While high-resolution structures of isolated NR domains have revealed conserved motifs and activation helices, full-length structures remain scarce, largely due to the flexibility of the hinge region. However, functional studies have demonstrated that mutations or deletions within the hinge domain can disrupt ligand-dependent transcriptional activity, even when ligand and DNA binding remain intact (Khan & Okafor, 2022). These findings imply that the hinge plays an active role in transmitting conformational information between the LBD and DBD.

Mechanistic Models of Communication

Several models have been proposed to explain how NRs like TR α coordinate activity across their domains. In the “induced fit” model, ligand binding stabilizes a specific LBD conformation that propagates through the hinge to reposition the DBD (Shang & Kojetin, 2021; Zhou, 2010). Conversely, in a “dynamic ensemble” model, the receptor samples multiple conformations, and ligand or DNA binding biases this ensemble toward transcriptionally active or repressive states (Khan et al., 2022). High-resolution techniques like X-ray crystallography and cryo-electron microscopy (cryo-EM) have revealed key insights into the architecture of the LBD and DBD, the more structured domains of NRs. These studies have identified conserved structural motifs (e.g., the zinc finger in DBDs and the alpha-helical sandwich in LBDs) and clarified how ligand and DNA binding influence receptor dimerization, coactivator recruitment, and transcriptional activation (Luisi et al., 1991; Rastinejad et al., 2013). However, these techniques are limited in capturing the IDRs, hence leaving out the NTD and the hinge domain,

which are often essential for allosteric communication and functional diversity. IDRs lack stable secondary or tertiary structure in isolation and are often excluded from structural models due to their flexibility and heterogeneity, either through proteolytic trimming or poor electron density in crystallographic studies. This exclusion has major implications. The allosteric coupling between receptor domains often involves these disordered segments, particularly the AF-1 transactivation domain in the NTD and the flexible hinge that connects the DBD to the LBD. Because these regions are poorly resolved, structural models of NRs frequently fail to capture the dynamic, ligand- and DNA-dependent conformational changes that underlie allostery (Meijssing et al., 2009; Kumar & Thompson, 2012). Moreover, most crystal structures are solved in static conditions with isolated domains, limiting our understanding of how domain-domain communication occurs in the full-length receptor bound to both ligand and DNA. As a result, traditional structural snapshots may oversimplify receptor behavior, missing the dynamic interplay between structure, disorder, and function that governs NR-mediated gene regulation.

Emerging techniques such as NMR spectroscopy, small-angle X-ray scattering (SAXS), single-molecule FRET, and integrative modeling are increasingly used to study the flexibility and conformational ensembles of NRs. These approaches hold promise for capturing the allosteric transitions and multidomain interactions that are central to receptor signaling but invisible in static crystallographic views.

The Hinge Domain: Structure, Sequence, and Variability

The NR hinge domain is a short, structurally elusive region that connects the DBD to the LBD (Helsen & Claessens, 2014). Although it is often treated as a passive linker in structural models, emerging evidence suggests that the hinge plays a pivotal role in modulating receptor flexibility, domain positioning, and interdomain allosteric communication (Haelens et al., 2007;

Helsen et al., 2012). The hinge domain also contributes to nuclear localization, receptor stability, and interactions with chromatin and coregulators, underscoring its functional importance beyond mere spatial separation (Hazarika et al., 2024; Zwart et al., 2010). Unlike the well-conserved and structurally ordered DBD and LBD, the hinge domain is highly variable in both sequence and length across NR families (Helsen & Claessens, 2014; Weikum et al., 2018). In thyroid hormone receptor alpha (TR α), the hinge spans approximately 20–30 residues and includes basic and hydrophobic residues, potential post-translational modification sites, and a conserved nuclear localization signal (NLS)(Anyetei-Anum et al., 2018; Helsen & Claessens, 2014). Sequence alignments show that while motifs such as the NLS are preserved, the surrounding residues exhibit significant variability, even among closely related receptors like TR β and RAR (Helsen & Claessens, 2014). The disordered nature of the hinge creates a challenge in resolving full-length NR structures using crystallography, which favors rigid and stable regions. In nearly all solved structures of TR α , the hinge is either absent or partially modeled, indicating conformational heterogeneity or structural flexibility (Rastinejad, 2025). Disorder prediction algorithms consistently assign the hinge region a high disorder propensity, and ensemble-based techniques such as small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) suggest that it exists in a range of extended or dynamic conformations rather than a single stable structure (Shamilov & Aneskievich, 2019; Stein & Schoonjans, 2015).

While the structured domains (LBD and DBD) have been well characterized, increasing evidence highlights the importance of the N-terminal domain and the linker or “hinge” region that connects these LBD and DBD (Arao & Korach, 2021). These flexible regions are less conserved among NRs and frequently lack a defined secondary or tertiary structure, exhibiting hallmarks of intrinsically disordered regions (IDRs) (Zanotti, 2023). They modulate important

ligand-dependent and -independent transcriptional activity of NRs (Kumarasamy et al., 2015; Rochel et al., 2011; Weigel & Zhang, 1998), and are increasingly recognized as key determinants of NR behavior (Uversky, 2024), facilitating structural adaptability (Zanotti, 2023), mediating interactions with coregulators (Zhang et al., 2024), and influencing nuclear localization (Li et al., 2025). Experimental evidence from deletion mutants, domain-swapping studies, and synthetic linkers indicates that subtle changes in hinge properties can lead to significant alterations in transcriptional response and ligand sensitivity (Bjarnason, 2024; Goto et al., 2024). Comparative analyses across the nuclear receptor superfamily reveal that hinge domains are not only variable in sequence but also in function (Helsen & Claessens, 2014). For example, the hinge in GR contributes to chromatin interactions and cofactor selectivity (Weikum et al., 2017), while in the ER, it plays a role in dimerization and DNA binding specificity (Mohideen-Abdul et al., 2017). This variability suggests that while the hinge is structurally disordered, it is evolutionarily tailored to the regulatory needs of each receptor.

Proposed Functions of the Hinge in Allosteric Regulation

Emerging evidence suggests that the hinge domain functions not only as a passive connector of the DBD and LBD, but also as a dynamic conduit that enables bidirectional transmission of structural signals across the receptor (Hazarika et al., 2024). This positioning allows the hinge to influence domain alignment, conformational flexibility, and ultimately, the receptor's ability to interpret ligand and DNA signals in a coordinated manner (Hazarika et al., 2024; Rastinejad, 2025). In thyroid hormone receptor alpha (TR α), the hinge domain appears to play a particularly important role in facilitating transitions between inactive and active receptor states (Brtko, 2021). The ability of TR α to function as both a transcriptional repressor in the absence of ligand and an activator upon ligand binding implies a high degree of conformational

plasticity (Vallortigara et al., 2009; Weikum et al., 2017). Studies suggest that the hinge domain permits the relative motion of the DBD and LBD, acting as a central pivot point within an ensemble of dynamic conformations (Chakrabarti & Chakravarty, 2022). This flexibility enables ligand-induced structural changes at the LBD to be transmitted through the hinge to reposition the DBD on DNA, and conversely, allows DNA binding to influence the conformation of the LBD (Rastinejad, 2025). Such bidirectional allostery is supported by experimental models in which hinge modifications impair transcriptional activity without affecting ligand or DNA binding directly (Fernandez, 2018; Yu et al., 2025). Beyond its mechanical role, the hinge may also serve as a regulatory interface. Post-translational modifications such as phosphorylation or acetylation within this region could dynamically alter the hinge's flexibility or interaction with other domains, effectively gating the flow of allosteric signals (Haelens et al., 2007; Hazarika et al., 2024; Rastinejad, 2025; Zwart et al., 2010). Moreover, domain-swap experiments (GLASS et al., 1991; Hollenberg et al., 1987) and synthetic hinge substitutions (Bridgham et al., 2006; Lee & Privalsky, 2005) have demonstrated that even subtle changes in hinge sequence can alter ligand sensitivity and DNA selectivity, highlighting its role in fine-tuning conformational coupling. Together, these observations position the hinge domain as a structurally disordered but functionally essential element that modulates interdomain communication in NRs, with implications for both receptor specificity and regulatory control (Rastinejad, 2025).

Understanding the role of the hinge domain in nuclear receptor allostery requires experimental approaches capable of capturing structural flexibility, transient interactions, and conformational transitions, supplementing the traditional high-resolution techniques which may lack these features. X-ray crystallography has provided detailed atomic-level views of the DBD and LBD; the hinge region is typically absent or unresolved due to its intrinsic disorder.

Solution-based techniques such as small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) have offered ensemble-level insights into global receptor conformation and local flexibility, yet they are limited by resolution and molecular size constraints (Gomes et al., 2020). Emerging cryo-electron microscopy (cryo-EM) approaches hold promise for characterizing full-length receptors in multiple states, though their application to NRs remains limited (Rastinejad, 2025). Together, these structural methods frame the hinge as a dynamic, unstructured element that resists static representation but may play a central role in domain coordination. To address these limitations, single-molecule and chemical biology tools offer complementary, dynamic perspectives. Single-molecule Förster Resonance Energy Transfer (smFRET) enables real-time monitoring of interdomain distances, capturing hinge-mediated transitions that reflect allosteric signal propagation (Chowdhury et al., 2024; Lamichhane, 2025). In combination with time-resolved and ensemble FRET methods, these tools allow the conformational landscape of NRs to be mapped under different ligand and DNA conditions. Together, they offer a powerful experimental platform to directly test how the hinge contributes to interdomain communication, advancing a more complete model of nuclear receptor signaling.

Thyroid Receptor Alpha as a Model System

Two thyroid hormone receptor (TR) proteins have been described, encoded by two closely related genes TR α and TR β (Thompson et al., 1987). The TR α gene, located on human chromosome 17, transcribes into three mRNA species, TR α 1, and TR α 2 and TR α 3, with TR α 2 and TR α 3 being truncated, less active forms of TR α 1 (Ortiga-Carvalho et al., 2014). TR α (with IUPHAR/BPS nomenclature NR1A1) (Alexander et al., 2019) is mostly expressed in the brain and is associated with the development of the nervous system. Triiodothyronine, T3 is the

agonistic ligand for of TR. Unlike other NRs, TR α is constitutively localized within the nucleus where it interacts with nucleosomal DNA (Wong et al., 1995).

TR α is a classic type II nuclear receptor that heterodimerizes with retinoid X receptor (RXR) and binds DNA in the absence of ligand, repressing target genes through recruitment of corepressors (Anyetei-Anum et al., 2018). Upon binding its ligand, T3, TR α undergoes a conformational change that results in corepressor release, coactivator recruitment, and transcriptional activation (Brtko, 2021). This switch involves structural rearrangements in the LBD that must be transmitted across the hinge domain to influence the DBD and its interactions with DNA (Helsen & Claessens, 2014). High-resolution structures of isolated TR α domains have revealed conserved motifs and activation helices (Nascimento et al., 2006; Ye et al., 2003), full-length structures remain scarce, largely due to the flexibility of the hinge region. However, functional studies have demonstrated that mutations or deletions within the hinge domain can disrupt ligand-dependent transcriptional activity, even when ligand and DNA binding remain intact (Miyamoto et al., 2001). These findings imply that the hinge plays an active role in transmitting conformational information between the LBD and DBD.

In TR α , the contribution of the hinge domain to this conformational landscape has yet to be directly visualized. Its highly disordered and variable sequence provides both flexibility and transient interaction surfaces necessary for signal transmission (Anyetei-Anum et al., 2018). Understanding how the hinge mediates interdomain coupling is therefore key to deciphering how TR α and other NRs integrate complex cellular signals to regulate gene expression precisely.

Single-molecule studies, including smFRET, increasingly support the latter view, revealing that NRs exhibit rapid, reversible transitions between conformations even under steady-state conditions (Paakinaho et al., 2017). This dynamic behavior suggests that

interdomain communication is not a simple switch but a tunable process modulated by multiple inputs. In TR α , the hinge's variability may reflect its dual role in maintaining flexibility for domain communication and enforcing structural constraints for effective DNA targeting and transcriptional output. This balance between disorder and function makes the hinge a unique regulatory element within the NR architecture. Site-specific labeling via unnatural amino acid incorporation (Chatterjee et al., 2013) and strain-promoted click chemistry (Kwon & Yang, 2017) ensures precise placement of donor and acceptor dyes on full-length TR α , preserving native folding and function.

CHAPTER THREE

MATERIALS AND METHODS

Materials

All chemicals and reagents used in this study were of analytical grade or higher and were used without further purification unless otherwise specified. Standard molecular biology supplies and equipment were sourced from commercial suppliers as indicated.

Plasmids and Strains

Expression constructs were based on custom pET-derived vectors containing amber (TAG) mutations at defined labeling positions within the target protein. The suppression system used for unnatural amino acid incorporation consisted of the pEVOL-pAzF plasmid (Addgene plasmid #31186), which encodes the orthogonal *Methanococcus jannaschii* aminoacyl-tRNA synthetase/tRNA pair specific for p-azido-L-phenylalanine (pAzF). Protein expression was carried out in *Escherichia coli* BL21(DE3) (New England Biolabs). For cell-free protein synthesis (CFPS) applications, S30-T7 extract was prepared from BL21(DE3) cells co-transformed with the expression and suppression plasmids.

Unnatural Amino Acids and Fluorophores

p-Azido-L-phenylalanine (pAzF) was obtained from Chem-Impex International and freshly dissolved in 0.1 M NaOH prior to use. Fluorophore labeling was performed using DBCO-Cy3 and DBCO-Cy5 (Lumiprobe), which were prepared in ddH₂O and anhydrous DMSO, respectively, at 10 mM stock concentrations and stored at −20 °C until needed.

Media, Antibiotics, and Inducers

Bacterial cultures were grown in Luria-Bertani (LB) broth or on LB agar plates (Miller formulation, BD Difco). Ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) were used for

plasmid selection. Protein expression was induced using 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; GoldBio) and 0.02% L-arabinose (Sigma), prepared as 1 M and 20% (w/v) stocks, respectively.

Buffers and General Reagents

Common buffer components including HEPES, Tris-HCl, NaCl, imidazole, DTT, and EDTA were purchased from Sigma-Aldrich or Thermo Fisher Scientific. Phenylmethylsulfonyl fluoride (PMSF) was used as a protease inhibitor at 1 mM final concentration, prepared as a 100 mM stock in isopropanol and added fresh to lysis buffers. For click-labeling reactions, proteins were buffer-exchanged into 20 mM Tris HCl, 150 mM NaCl, pH 7.5.

Protein Purification

His-tagged proteins were purified using Ni-NTA agarose resin (Fisher Scientific) under native conditions. Protein samples were concentrated and desalted using Amicon Ultra centrifugal filters (10 kDa MWCO, Millipore). Size-exclusion chromatography (SEC) was performed using a Superdex 75 Increase 10/200 GL column (Cytiva) operated on an ÄKTA Pure system.

Western Blotting Reagents.

Protein detection was performed using 10–12% SDS-PAGE gels (homemade or Bio-Rad pre-cast) and nitrocellulose membranes (0.45 μ m pore size, Millipore). Primary antibodies included mouse anti-His tag (1:2,000; Thermo Fisher). HRP-conjugated secondary antibodies, and goat-anti-mouse (1:5,000; Bio-Rad) were used for chemiluminescent detection. Biorad equipment

Methods

Preparation of mutTR construct

The mutTR (TR2UAA) construct was generated from the parental plasmid cTRA1_TEV (Appendix 1.1) using site-directed PCR mutagenesis. Two amber stop codons were introduced:

one at position 126 in the DBD, replacing the codon for aspartic acid (GAC) with TAG, and another at position 190 in the LBD, replacing the codon for leucine (CTG) with TAG. Mutagenic primers were designed to introduce these substitutions, and PCR was carried out using a high-fidelity DNA polymerase. Following amplification, the parental plasmid template was digested with DpnI to eliminate methylated, non-mutated DNA. The resulting PCR product was transformed into chemically competent *E. coli* cells, and positive colonies were identified via colony PCR and confirmed by Sanger sequencing. The final construct, containing both amber mutations, was designated mutTR (TR2UAA).

An alternative construct was designed targeting positions 41 (DBD) and 151 (LBD), replacing a Tyr codon (TAC) and a Met residue (ATG), respectively, with the amber stop codon TAG using site-directed PCR mutagenesis. See sequence 1.2 in *Appendix*. The resulting plasmid was verified by sequencing and designated as the revised mutTR – TRmutYM – construct for subsequent expression and labeling experiments. Mutagenic primers for Y2TAG, 5' TCC TGC AAG TAG GAT GGG TGC 3' and 5' GCA CCC ATC CTA CTT GCA GGA 3'; for M2TAG, 5' CAG TCG CCC TAG GCC TCC ATG 3' and 5' CAT GGA GGC CTA GGG CGA CTG 3'.

Protein expression and purification

Cell-Free Protein Synthesis (CFPS)

Cell-free protein synthesis was used to express site-specifically labeled constructs containing amber codons for unnatural amino acid incorporation. Reactions were performed using an S30-T7 *E. coli* extract prepared in-house, optimized for orthogonal translation systems.

Preparation of S30-T7 Lysate

E. coli BL21(DE3) cells were transformed with the pEVOL-pAzF plasmid, which carries chloramphenicol resistance (Cam^R) for selection. Glycerol stocks of the transformed strain were prepared and stored for future use. A single colony from the transformation plate was used to inoculate 5 mL of LB medium and cultured overnight at 37°C with shaking at 250 rpm. The overnight culture was diluted 1:50 into 500 mL to 1 L of 2xYT media and grown at 37°C until reaching an optical density (OD₆₀₀) of approximately 1. At this point, expression was induced by the addition of sterile arabinose to a final concentration of 0.1% (v/v) or higher, and 1 mM IPTG. Following induction, the culture was allowed to continue growing at 37°C until reaching an OD₆₀₀ of approximately 4. Cells were then harvested by centrifugation at $7,000 \times g$ for 10 minutes at 4°C. The resulting cell pellet was gently resuspended in an equal volume of ice-cold S30 lysate buffer (1:1 v/v), composed of 10 mM Tris-acetate (pH 7.4), 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM dithiothreitol (DTT), and 0.5 mL/L β -mercaptoethanol (β ME). After the initial wash, the cells were pelleted again and resuspended in 15 mL of fresh S30 lysate buffer. Cell lysis was carried out using a French press pre-cooled to 4°C, operated at a working pressure of 15,000 psi. Following homogenization, 100 μ L of 0.1 M DTT was added per 10 mL of lysate. The crude lysate was then clarified by centrifugation at either $24,700 \times g$ or $13,000 \times g$ for 30 minutes at 4°C. Aliquots of 200 μ L were flash-frozen in liquid nitrogen and stored at -80°C for subsequent use in *in vitro* translation reactions.

Cell-Free Protein Synthesis (CFPS) Using the S30-T7 Lysate

In vitro protein synthesis reactions were conducted using the prepared S30-T7 lysate. Reaction mixtures were assembled in 1.5 mL microcentrifuge tubes by combining the following components to achieve the final concentrations:

- 55 mM HEPES-KOH (pH 8.0), 14 mM magnesium acetate, 50 mM potassium acetate, 155 mM ammonium acetate, 3% (w/v) polyethylene glycol, and 40 mM D-(-)-3-phosphoglyceric acid disodium salt;
- 2.5 mM premixed 20 canonical amino acids
- 1.2 mM ATP, 1 mM GTP, and 0.8 mM UTP;
- 30% (w/v) S30-T7 lysate
- DNase, RNase free H₂O to reaction volume
- 1 mM IPTG;
- 2.5 mM *p*-azido-L-phenylalanine (pAzF);
- 10 µg/mL of the RIPL plasmid DNA and 10 µg/mL of the mutTR DNA template.

Lysate was then diluted with buffer as needed to achieve a final protein concentration of 22 mg/mL. The complete reaction mixture was prepared by combining the lysate with the reagent mix in a 30:70 (v/v) ratio (lysate to buffer/reagents). Final volumes were adjusted with autoclaved, DNase- and RNase-free water as required. Reactions were incubated at 37°C for 2 hours with vigorous shaking at 1200 rpm. Alternatively, reactions could be performed overnight at 20°C to assess the impact of lower temperature incubation on protein yield. Protein expression was assessed via western blotting using an anti-His₆ antibody. The protein concentration was measured via the Bradford Assay (Olson & Markwell, 2007).

***E. coli* Protein Expression**

The mutTR expression construct and the pEVOL-pAzF plasmid (Addgene #31186), which harbors the orthogonal *Methanococcus jannaschii* tRNA^{Tyr} and evolved aminoacyl-tRNA synthetase, were co-transformed into *E. coli* BL21(DE3) competent cells. Cells were plated on LB-agar containing 100 µg/mL ampicillin and 25 µg/mL chloramphenicol for selection of both plasmids. For protein expression, a single colony was used to inoculate a 10 mL LB starter

culture with appropriate antibiotics and grown overnight at 37°C. This was used to inoculate 500 mL of fresh LB medium containing 1 mM pAzF (Chem-Impex), freshly dissolved in 0.1 M NaOH and filter sterilized before addition. Cultures were grown at 37°C to an OD₆₀₀ of ~0.5–0.6, then supplemented with 0.02% L-arabinose to induce AzFRS expression and 0.5 mM IPTG to induce target protein expression. Expression continued for 16–20 hours at 20°C to promote soluble protein folding.

Protein Purification

Cells were harvested by centrifugation (4,000 × g, 15 min, 4°C), resuspended in lysis buffer [50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF], and lysed via sonication. Lysates were cleared by centrifugation (17,000 × g, 30 min, 4°C), and the supernatant was purified via Ni-NTA affinity chromatography under native conditions, followed by further purification using size-exclusion chromatography (SEC) to obtain monodisperse protein suitable for downstream biophysical and labeling applications. Elution was performed with 250 mM imidazole gradient in Tris buffer. Protein purity was assessed by SDS-PAGE, and concentration was determined via Bradford assay. Pooled fractions containing the target protein were concentrated using a centrifugal concentrator (Amicon Ultra, 10 kDa MWCO) and buffer-exchanged into SEC running buffer (2 mM Tris, 150 mM NaCl, pH 7.5) during concentration. Purified protein was aliquoted, flash-frozen in liquid nitrogen, and stored at –80°C. For short-term use, proteins were stored at 4°C with 5% glycerol added to maintain stability.

Unnatural Amino Acid Incorporation via Amber Codon Suppression

Unnatural amino acid incorporation was achieved through amber (UAG) codon suppression using an orthogonal tRNA/tRNA synthetase pair specific to para-azido-L-phenylalanine (pAzF). The gene of interest, TR construct, was engineered to contain two amber stop codons at desired

incorporation sites via site-directed mutagenesis (see Molecular Cloning section). Incorporation positions were selected based on solvent accessibility and functional neutrality. Successful incorporation of pAzF was confirmed by intact mass spectrometry and successful downstream labeling reactions (see smFRET Labeling section). No protein expression was detected in the absence of pAzF, validating site-specific suppression of the amber codon (Chatterjee et al., 2013).

Protein labeling - Click chemistry

Site-specific labeling of recombinant proteins with Cy3 and Cy5 fluorophores was achieved via strain-promoted azide–alkyne cycloaddition (SPAAC) between para-azido-L-phenylalanine (pAzF) and dibenzocyclooctyne (DBCO)-conjugated dyes. This enabled single-molecule FRET studies on conformational dynamics. Purified protein containing site-specifically incorporated pAzF at designated positions (**Figure 3.1**) was buffer-

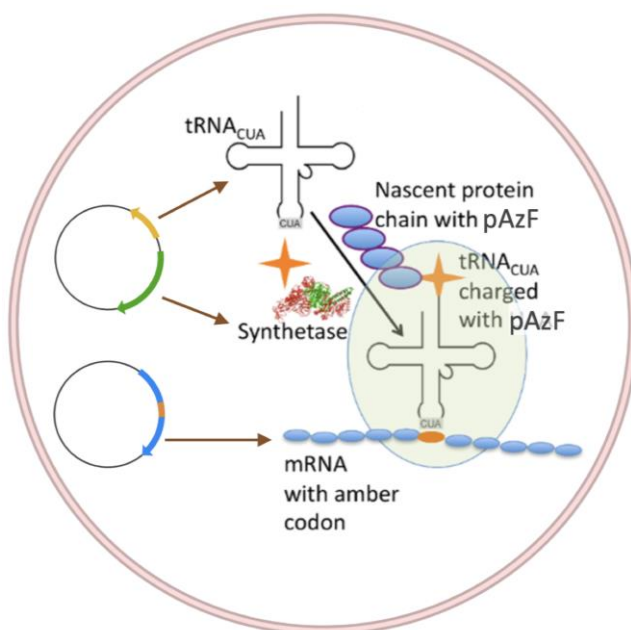


Figure 3.1. A schematic of UAA incorporation method: Plasmids encoding the suppressor tRNA_{CUA}, the evolved amino acyl-tRNA synthetase (aaRS) and the target protein with the amber codon are transfected into cells.

exchanged into labeling buffer (20 mM Tris, 150 mM NaCl, pH 7.5) using a PD-10 desalting column (GE Healthcare). The protein was concentrated to 20–50 μ M using a 30 kD centrifugal filter (Amicon Ultra, Millipore). Sulfocyanine-DBCO-Cy3 and sulfocyanine-DBCO-Cy5 (Lumiprobe) were prepared in ddH₂O and DMSO respectively at 10 mM stock concentrations. The protein was incubated with 1.5–2.5 molar equivalents of dye or at 4°C, rotating for 2-16 hours. Reactions were protected from light to minimize photobleaching. Excess free dye was

Degree of labeling (DOL) was estimated spectrophotometrically using a NanoDrop One. Absorbance at 280 nm, 550 nm (Cy3), and 650 nm (Cy5) was measured. Labeling efficiency was calculated using extinction coefficients:

- $\epsilon_{(280),\text{protein}} = [\text{insert value}]$
- $\epsilon_{(550),\text{Cy3}} = 150,000 \text{ M}^{-1}\text{cm}^{-1}$
- $\epsilon_{(650),\text{Cy5}} = 250,000 \text{ M}^{-1}\text{cm}^{-1}$

Corrections were applied for dye absorbance overlap at 280 nm. Labeling efficiency typically ranged from 70–90%. Labeled proteins were aliquoted, flash-frozen in liquid nitrogen, and stored at –80°C. All labeled proteins were handled under light-protective conditions to preserve dye integrity.

Single-molecule immobilization and smFRET

Single-molecule fluorescence measurements were carried out using a custom-built prism-type total internal reflection fluorescence (TIRF) microscope. The system was constructed around an Olympus IX73 inverted microscope and fitted with a 60 $\times$ water-immersion objective (1.49 NA, Olympus)(Liu et al., 2023). Quartz microscope slides (G. Finkenbeiner) and coverslips (Fisher Scientific, Cat. #1254588) were cleaned and PEG-passivated using a mixture of mPEG-SVA and biotin-PEG-SVA (Laysan Bio) to minimize non-specific adsorption. Flow

chambers were assembled using double-sided tape to sandwich the PEGylated slides and coverslips. After assembly into microfluidic flow chambers, the surface was incubated with 0.2 mg/mL NeutrAvidin (Thermo Fisher Scientific) in PBS for 10 minutes, followed by washing with imaging buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 2 mM Trolox). Biotinylated anti-His antibody was immobilized onto the surface via biotin-NeutrAvidin interactions, followed by washing with imaging buffer. The labeled mutTR was diluted to ~50–100 pM and immobilized onto the surface via Histag-antiHis antibody interactions. Excess unbound molecules were washed out before imaging. To minimize photobleaching and improve signal stability, an oxygen scavenging system containing 5 mM protocatechuic acid (Sigma, Cat. #37580) and 3 U/mL protocatechuate-3,4-dioxygenase (Oriental Yeast) was added to the chamber prior to imaging.

Fluorophores were excited using a 532 nm laser (CrystaLaser), and emission from both donor and acceptor channels was captured using an EMCCD camera (Andor Technology) with a frame rate of 100 ms per image. Data were acquired using a custom single-molecule acquisition interface. Time-resolved fluorescence trajectories were analyzed using IDL-based scripts provided by Dr. Taekjip Ha's lab (<http://ha.med.jhmi.edu/resources/>). All imaging experiments were conducted at room temperature.

Raw fluorescence time traces were extracted using custom MATLAB scripts or ImageJ-based FRET analysis software. Background subtraction and correction for donor leakage, direct excitation, and γ -factor were performed before FRET efficiency (E_{FRET}) was calculated as:

$$E_{\text{FRET}} = I_A / (I_D + I_A)$$

where I_D and I_A are the background-corrected donor and acceptor intensities, respectively. Only molecules showing clear single-step photobleaching in both channels were included in the analysis. Hidden Markov Modeling (HMM) or thresholding approaches were used to identify

transitions between conformational states. Anticorrelated donor and acceptor intensity changes were used as evidence of FRET transitions.

CHAPTER FOUR

RESULTS AND DISCUSSION

Incorporation of Unnatural Amino Acids via Amber Suppression

To enable site-specific labeling, p-azido-L-phenylalanine (pAzF) was incorporated at designated positions within the mutTR sequence using amber codon suppression. Constructs containing TAG codons at residues 126 and 190 were co-expressed with the pEVOL-pAzF system in *E. coli* BL21(DE3) and in cell-free reactions. In the absence of pAzF, no full-length protein was observed, confirming that amber suppression was required for translation. Upon supplementation with 1 mM pAzF, full-length protein (molecular weight, MW = 48 kD) was produced, indicating successful suppression and incorporation of the unnatural amino acid (**Figure 4.1**). Truncated proteins were produced as well at MW of 28 kD, 22.4 kD, and 13 kD. This indicates that there were instances where *E. coli* translated the incorporated amber stop codons as stop codons and terminated translation instead of incorporating the pAzF amino acid.

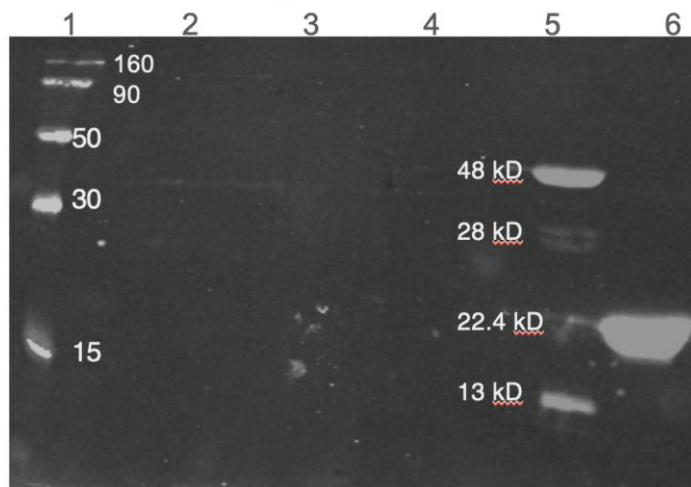


Figure 4.1 Western blot analysis of an overexpression test of mutTR. mutTR constructs were co-expressed with pEVOL-pAzF. Full-length protein and truncated proteins were expressed. Lane 1 = MW ladder, Lane 5 = mutTR, Lane 6 = positive control.

Both *E. coli* and CFPS reactions yielded sufficient soluble protein (approx. 3 $\mu\text{g/mL}$ per 1 L of culture for *E. coli* expression) for downstream labeling and functional studies. The soluble fraction was confirmed by Ni-NTA purification and Coomassie-stained gels, which showed high purity (>70%) of eluted protein.

Site-Specific Labeling with Cy3 and Cy5 Fluorophores

Labeling was carried out using DBCO-Cy3 and DBCO-Cy5 to target the azide group of pAzF via SPAAC click chemistry. After labeling and purification, UV-Vis spectroscopy revealed strong absorbance peaks at 550 nm and 650 nm, confirming successful attachment of Cy3 and Cy5, respectively (**Figure 4.2**). The degree of labeling (DOL) was estimated to be ~0.9 for each dye, indicating near-stoichiometric conjugation. Size-exclusion chromatography confirmed separation of labeled protein from unreacted dye.

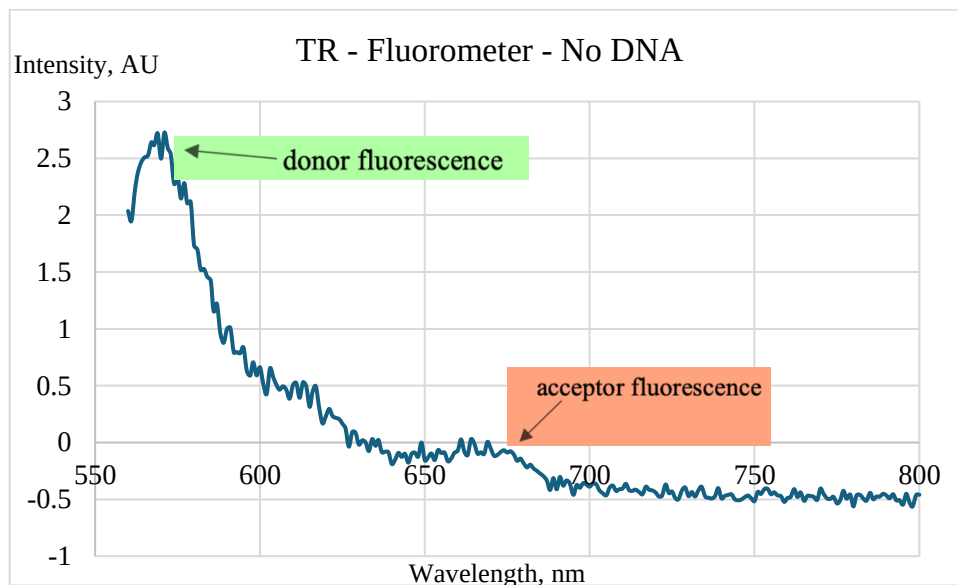


Figure 4.2 Bulk fluorescence measurements of labeled mutTR after removing excess fluorophores. This sample contained labeled mutTR without DNA and was excited at the donor excitation wavelength of 532 nm. The donor fluorophore Cy3 emitted at ~575 nm and the acceptor fluorophore Cy5 emitted at ~675 nm. This is indicative of energy transfer.

Interdomain FRET Occurs in mutTR

Single-molecule FRET analysis was used to examine intramolecular distance changes and conformational dynamics of labeled mutTR. Immobilized Cy3/Cy5-labeled molecules were imaged using TIRF microscopy under oxygen-scavenging conditions. Several FRET experiments hardly produced anti-correlated donor and acceptor intensity fluctuations which are usually indicative of dynamic FRET transitions, making it difficult to obtain useful trajectories for FRET efficiency histograms. However, one mutTR molecule (in the absence of DNA) revealed anti-correlated donor and acceptor intensity fluctuations indicative of dynamic FRET transitions (**Figure 4.3**). This data was obtained from a single molecule and serves as preliminary rather than conclusive data. The E_{FRET} trajectory showed a low FRET state of 0.33 and a high FRET state of 0.86. These data suggest that mutTR with or without DNA exists in multiple conformational states, rather than a fixed open or closed conformation under equilibrium conditions. The high FRET value corresponds to a distance of 40 Å between the fluorophores while the low FRET value corresponds to a distance of 61 Å. This suggests that the receptor is dynamic even in the absence of DNA. More data will be collected to investigate this phenomenon.

Redesigning mutTR

After several troubleshooting experiments yielding no useful data, the biophysical structure and construct design of mutTR was revisited and it was found that the one of the labeling sites was pointing inward toward the core of the protein (**Figure 4.4**). With this, a new TR construct, TRmutYM, was designed to incorporate the stop codons at Y43 and M151, where both residues have side chains that are not involved in major structural or functional interactions, and are accessible for labeling (**Figure 4.5**).

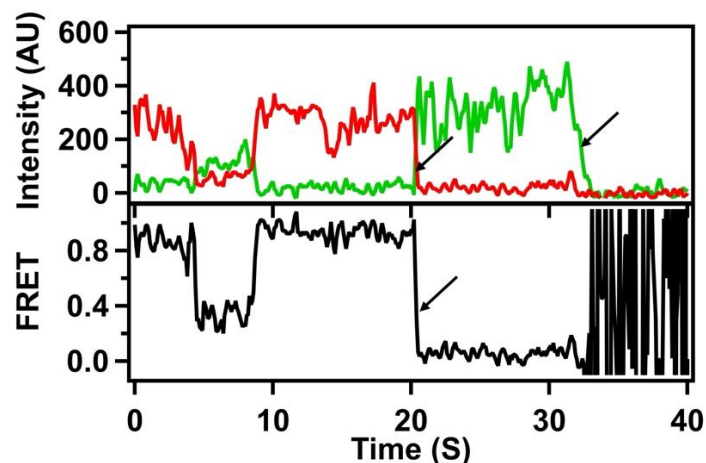


Figure 4.3 Single-molecule intensity time trajectories (top: donor green; acceptor red) and FRET efficiency (bottom) for individual TR (no DNA bound). Black arrows represent single-step photobleaching events. These traces were obtained from a single immobilized TR molecule (one molecule out of 91). Two FRET states were observed, a high FRET of 0.86 and a low FRET of 0.33, showing both the open and closed conformations.

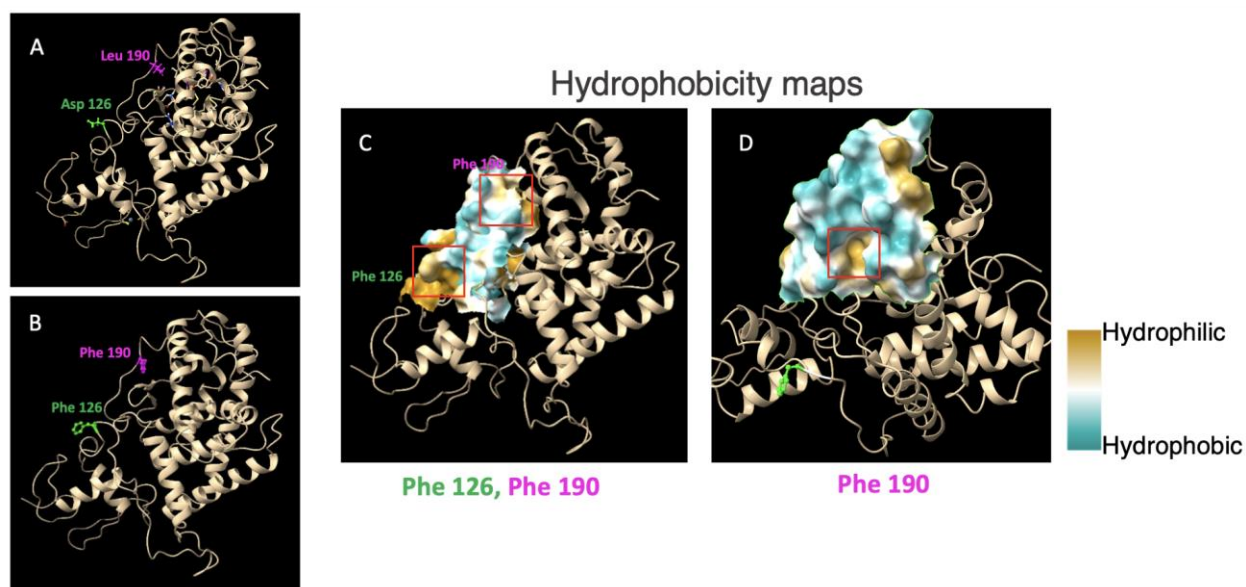


Figure 4.4. Analysis of mutTR (without) tertiary structure in ChimeraX. **A.** mutTR structure showing the side chains of D126 (green) and L190 (pink). **B.** D126 and L190 have both been swapped for Phe to mimic a pAzF side chain. F190 points inward toward the core of the protein and seems inaccessible. **C.** and **D.** Hydrophobicity maps of the zone around F126 and 190. Even though both have hydrophilic side chains, F190 is embedded and cannot interact in labeling reactions (**D**).



Figure 4.5. Analysis of TRmutYM tertiary structure in ChimeraX. This analysis used the same starting structure as Figure 4.4. It is rotated $\sim 90^\circ$ along the vertical axis. **A.** TRmutYM structure showing the side chains of Y43 (cyan) and M151 (pink). **B.** Y43 and **C.** M151 have both been swapped for Phe to mimic a pAzF side chain. Both Phe side chains orient outward from the core of the protein structure and are accessible for labeling. **D.** Hydrophobicity maps of the zones around F43 and F151. Both residues have hydrophilic side chains and are readily available for labeling reactions.

Similar troubleshooting as was done for the mutTR were performed for TRmut YM as well.

Steps to enhance labeling included incubating the Cy5 with the protein for 4 hours before adding the Cy3, linearizing the labeled protein for bulk fluorescence measurements, varying the fluorophore ratios and incubation times. The distance between the labeling sites were measured in ChimeraX to be ~ 27 Å without DNA and ~ 54 Å with DNA. Despite these measurements, the labeled molecules did not yield any FRET trajectories with anticorrelation. This still leaves the reactivity of Cy5.

These observations, though preliminary, suggest that the structural changes that occur in TR are transient and dependent on DNA binding. However, rather than having one distinct conformation correlate to a DNA-bound state, it is more of a tapping motion that closes and releases in an unstable manner, with DNA binding increasing stability of TR. This dynamic behavior has been described by Paakinaho and colleagues (Paakinaho et al., 2017).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

We have shown that interdomain communication does occur between the DNA-binding and ligand-binding domains of the thyroid hormone receptor. This conclusion, however, is preliminary, as the current data are insufficient to fully support the claim. We faced significant challenges with sample preparation, right from our construct design all the way to fluorophore labeling. A major challenge was with the protein structure: one labeling site, PheAz202, was oriented inward toward the core of the mutTR protein, preventing the attachment of the sulfo-cyanine fluorophore. This was resolved when we created a newer construct, TRmutYM, which had both fluorophore attachment sites oriented outward, making both PheAz sites readily available for labeling. We were also challenged by the labeling chemistry of our fluorophores. Sulfo-cyanine 3 DBCO quickly attached to the binding sites, whereas sulfo-cyanine 5 DBCO did not. We performed multiple troubleshooting experiments to optimize the labeling conditions and improve data reproducibility. In the end, we found out that Cy5 had variable solubility and shelf life compared to Cy3. This suggests that the labeling conditions and time be varied for the fluorophores to enhance even donor:acceptor fluorophore attachment ratios. Further work must be done to establish a protocol for TRmutYM labeling, considering fluorophore ratios and labeling conditions and time. Additionally, more smFRET data must be collected to enhance reproducibility and confidence in the proposed conclusions. Since smFRET sample preparation requires antibody-specific binding to a quartz slide, several rounds of purification may be avoided, improving total protein yield.

Further research must be done to understand the structural perspective of this interdomain interaction. It is important to determine what amino acid residues are involved in the crosstalk,

what bond or interaction types are present, if any new interactions are established, and what forces drive these interactions. To better understand the role of the hinge domain, it is also important to study the impact of other interacting partners of the thyroid hormone receptor. In this study, we observed the difference in the ligand-bound receptor in the presence and absence of DNA. Other binding partners to be studied include varying the presence of the ligand (T3), the heterodimerization partner RXR and its ligand (9c). Each of these conditions could also be tested in the presence and absence of DNA. There is also the impact of other members of the pre-initiation complex, especially ones that interact with the hinge of nuclear receptors, such as coactivators interacting with the LXXLL motif on the hinge domain of some receptors.

In addition, other powerful spectroscopic techniques like nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) can be used to study structural changes in these nuclear hormone receptors, offering complementary insights into protein dynamics and interactions. NMR provides high-resolution structural information and can detect subtle conformational changes, while EPR is particularly useful for studying long-range distances and local environment changes around specific sites. NMR is restricted to smaller proteins (typically under 50 kDa) due to spectral crowding and line broadening in larger systems. This implies the requirement of a high-field magnetic source (for example, 1200 Hz – 1500 Hz) for our 48 kDa TRmutYM protein and its complexes. Advances in protein deuteration and selective labeling strategies may also extend the size range for NMR studies. Secondly, NMR requires milligram amounts of pure, homogeneous sample and extended measurement times. Our *E. coli* BL21(DE3) cells have produced 1-3 mg of TRmutYM protein per liter of culture. With such low yield, relatively more resources will be required to produce the required amount of pure sample needed for NMR. However, the culture conditions can be varied for optimal protein expression

to increase yield. For EPR, while less size-limited, it often necessitates site-specific spin labeling. This allows labeling both fluorophore attachment sites with the same label, minimizing the unfavorable effects of Cy5 binding in the smFRET experiments. Developing new spin labels and pulse sequences could also enhance sensitivity and information content. Both methods demand expertise in data interpretation and may require computational modeling to extract meaningful structural information. Combining NMR and EPR data with complementary methods like smFRET and computational modeling could provide comprehensive structural insights.

Lastly, it is important to ensure the biological relevance of these structural data. *In cellulo* FRET assays can detect conformational changes in real-time within cells. This allows researchers to monitor interdomain dynamics under physiological conditions, while also varying the presence and absence of binding partners listed earlier. Other cell-based assays can also be used to assess ligand binding, receptor activation, and downstream signaling events. Physiologically relevant hinge mutations can be predicted from MD simulations and smFRET experiments and introduced into the hinge to vary rigidity, based on a scale proposed by bioinformatics studies (Huang & Nau, 2003). The role of hinge flexibility will be determined by transcriptional activity. Subsequently, these data will be compared with naturally occurring hinge mutations. Reporter gene assays could also be employed to measure receptor activity. Here, multiple complexes can be tested with the mutant (hinge) TRmutYM. In these assays, cells are transfected with a plasmid containing a receptor-responsive promoter linked to a reporter gene, such as luciferase. Upon receptor activation, the reporter gene is expressed, and its activity can be quantified. While cell-based assays offer valuable insights, they also have limitations. Results can be influenced by cell type, expression levels of transfected proteins, and potential artifacts from protein labels. Careful assay optimization and appropriate controls are crucial for obtaining

reliable data. Future developments in cell-based assays may include the integration of advanced imaging techniques for improved spatial and temporal resolution of receptor dynamics.

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APPENDIX

1.1 cTRA_TEV

AGCAGCGGCGAGAATCTTTATTTTCAGGGCCATATGATGGAACAGAAACCTAGCACCCCTGGATCC
GCTGTCTGAACCGGAAGATACCCGCTGGCTGGACGGCAAACGTAAACGTAAATCCTCCCAGTGCC
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AAGGTTGCTGCATGGAATCATGTCTCTGCGTGCGGCTGTTTCGTTACGACCCGGAAAGCGAAACC
CTGACCCTGTCTGGCGAAATGGCTGTTAAACGTGAACAGCTGAAAAACGGTGGCCTGGGTGTTGT
TTCTGATGCTATCTTCGACCTGGGCAAGTCTCTGTCCGCATTCAACCTGGATGATACTGAGGTGGC
ACTGCTGCAAGCCGTGCTGCTGATGTCTTCCGACCGCACGGGCCTGATCTGTGTTGACAAAATCGA
AAAGTGCCAAGAGACTTATCTGCTGGCATTGTAACATTACATCAACTATCGTAAACACAACATTCC
GCATTTCTGGCCAAAGCTGCTGATGAAAGTTACCGATCTGCGTATGATTGGTGCCTGCCACGCGTC
TCGTTTCCTGCATATGAAAGTTGAATGTCCAACCGAACTGTTCCCGCCGCTGTTCTGGAAGTATT
CGAAGATCAGGAAGTTTGA

1.2 cTRA_DL_2UAA_TEV (mutTR DBD-LBD)

AGCAGCGGCGAGAATCTTTATTTTCAGGGCCATATGATGAGCGGTTACATCCCGTCCTACCTGGAC
AAAGACGAACAGTGCGTGTTGTGGCGACAAAGCTACCGGTTATCACTACCGCTGCATCACCTG
CGAAGGCTGTAAAGGTTTCTTCCGCCGTACTATCCAAAAAACCTGCATCCGACCTACTCCTGTAA
ATACGACGGCTGCTGTGTTATCGACAAAATCACGCGTAACCAAGTGTACAGCTGTGCCGTTTTAAAAA
ATGCATCAGCGTTGGCATGGCTATGGATCTGGTTCTGGACTAGAGCAAACGTGTTGCGAAACGTA
AGCTGATCGAAGAAAACCGTGAACGTGCTCGTAAGGAGGAAATGATCAAATCTCTGCAGCACCGT
CCATCTCCAAGCGCAGAGGAGTGGAACTGATCCATGTGGTGACCGAAGCTCATCGTTCCACCAA
TGCCCAAGGCTCCCATTTGGAAACAAAAACGTAAATTCTAGCCAGAAGACATCGGTGAGTCCCCGA
TGGCTTCCATGCCGGATGGTGACAAAGTTGATCTGGAAGCATTCTCCGAATTCATAAAATCATTA
CCCCTGCTATTACCCGTGTTGTGGACTTCGCGAAAAAACTGCCAATGTTTTCTGAGCTGCCTTGC
AGGATCAGATTATCCTGCTGAAAGGTTGCTGCATGGAATCATGTCTCTGCGTGCGGCTGTTTCGTT
ACGACCCGGAAAGCGAAACCCTGACCCTGTCTGGCGAAATGGCTGTTAAACGTGAACAGCTGAAA
AACGGTGGCCTGGGTGTTGTTTCTGATGCTATCTTCGACCTGGGCAAGTCTCTGTCCGCATTCAAC
CTGGATGATACTGAGGTGGCACTGCTGCAAGCCGTGCTGCTGATGTCTTCCGACCGCACGGGCCTG
ATCTGTGTTGACAAAATCGAAAAGTGCCAAGAGACTTATCTGCTGGCATTGTAACATTACATCAAC
TATCGTAAACACAACATTCCGCATTTCTGGCCAAAGCTGCTGATGAAAGTTACCGATCTGCGTATG
ATTGGTGCCTGCCACGCGTCTCGTTTCCTGCATATGAAAGTTGAATGTCCAACCGAACTGTTCCCG
CCGCTGTTCTGGAAGTATTGGAAGATCAGGAAGTTTGA

1.3 cTRA_DLYM_2UAA_TEV (TRmutYM DBD-LBD)

AGCAGCGGCGAGAATCTTTATTTTCAGGGCCATATGATGGAACAGAAACCTAGCACCCCTGGATCCGCT
GTCTGAACCGGAAGATACCCGCTGGCTGGACGGCAAACGTAAACGTAAATCCTCCCAGTGCTGGTGA

AATCCTCCATGAGCGGTTACATCCCGTCCTACCTGGACAAAGACGAACAGTGCGTGGTGTGTGGCGAC
AAAGCTACCGGTTATCACTACCGCTGCATCACCTGCGAAGGCTGTAAAGGTTTCTTCCGCCGTACTATC
CAAAAAAACCTGCATCCGACCTACTCCTGTAAATACGACGGCTGCTGTGTTATCGACAAAATCACGCG
TAACCAGTGTGAGCTGTGCCGTTTTTAAAAAATGCATCAGCGTTGGCATGGCTATGGATCTGGTTCTGGA
CGGCAGCAAACGTGTTGCGAAACGTAAGCTGATCGAAGAAAACCGTGAACGTCGTCGTAAGGAGGAA
ATGATCAAATCTCTGCAGCACCGTCCATCTCCAAGCGCAGAGGAGTGGGAACTGATCCATGTGGTGAC
CGAAGCTCATCGTTCCACCAATGCCCAAGGCTCCCATTGGAAACAAAAACGTAAATTCGGCCCAGAAG
ACATCGGTGAGTCCCCGATGGCTTCCATGCCGGATGGTGACAAAGTTGATCTGGAAGCATTCTCCGAA
TTCACTAAAATCATTACCCCTGCTATTACCCGTGTTGTGGACTTCGCGAAAAAACTGCCAATGTTTTCT
GAGCTGCCTTGCGAGGATCAGATTATCCTGCTGAAAGGTTGCTGCATGGAAATCATGTCTCTGCGTGC
GGCTGTTTCGTTACGACCCGAAAGCGAAACCCTGACCCTGTCTGGCGAAATGGCTGTAAACGTGAAC
AGCTGAAAAACGGTGGCCTGGGTGTTGTTTCTGATGCTATCTTCGACCTGGGCAAGTCTCTGTCCGCAT
TCAACCTGGATGATACTGAGGTGGCACTGCTGCAAGCCGTGCTGCTGATGTCTTCCGACCGCACGGGC
CTGATCTGTGTTGACAAAATCGAAAAGTGCCAAGAGACTTATCTGCTGGCATTGTAACATTACATCAA
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TGGTGCCTGCCACGCGTCTCGTTTCCTGCATATGAAAGTTGAATGTCCAACCGAACTGTTCCCGCCGCT
GTTCTGGAAGTATTTCGAAGATCAGGAAGTTGGCCTCGAGCACCACCACCACCACCTGAGATCCGG
CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC
CTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

VITA

Barbra Dompreeh was born in Tema, Ghana, in 1996. She earned a Bachelor of Science degree in Biochemistry, Cell and Molecular Biology from the University of Ghana, Legon, in 2017. After graduation, she worked as an administrative assistant and teaching assistant in the Department of Biochemistry, Cell, and Molecular Biology at the University of Ghana from 2017 to 2018. She then gained research experience as an intern at the Noguchi Memorial Institute of Medical Research in Accra, Ghana.

In August 2019, Barbra began her graduate studies at the University of Tennessee, Knoxville, in the Genome Science and Technology program, formerly the UT-ORNL Graduate School of Genome Science and Technology, now part of the UT-ORII Bredesen Center. During her time at UT, she conducted research in the Fernandez lab and served as a graduate teaching assistant for general biology and biochemistry.