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Characterization Of The Androgen Receptor H1-H3 Loop As A Putative Fkbp Regulatory Surface

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DEDICATION

This dissertation is dedicated to my family. I love you deeply with all my heart. To my son Luca, you are my light and motivation to be the better version of myself day to day. Always remember that I love you without measure. To my husband Aldo, for all your love and encouragement, your partnership and steadfastness sustain me. This accomplishment would not have been possible without you.

CHARACTERIZATION OF THE ANDROGEN RECEPTOR H1-H3 LOOP AS A PUTATIVE FKBP REGULATORY SURFACE

by

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ABSTRACT

Prostate Cancer (PCa) is one of the most common life-threatening malignancies diagnosed among American men. Initiation and progression of PCa are dependent upon androgen receptor (AR) regulated genes. Functional receptor conformation is influenced by the cooperation of chaperone and cochaperone proteins including the 52 and 51 kDa FK506 binding proteins (FKBP52 and FKBP51). FKBP52 is known for being a positive regulator of AR, PR (progesterone receptor), and GR (glucocorticoid receptor) activity, whereas FKBP51 negatively regulates steroid hormone receptor activity. As a result, these two proteins have become highly promising therapeutic targets for the disruption of mechanisms important in several endocrine-related diseases such as prostate cancer. Previous studies have identified the BF3 surface as an AR-specific regulatory site for FKBP52, and it is likely that there is another common FKBP regulatory surface among all the regulated receptors, like the H1-H3 loop. GR is known to be more hypersensitive to FKBP52 regulation, and mutations within the GR H1-H3 loop affect FKBP-mediated receptor activities. Thus, we conducted site-directed mutagenesis to identify the residues within the human AR H1-H3 loop that are critical for FKBP co-chaperone regulation. Taking advantage of the distinct GR hypersensitivity to FKBP52, two classes of functional mutants were generated to make the human AR H1-H3 loop more like human GR or guinea pig GR. In addition, yeast-based reporter assays were performed to assess the role and relevance of those mutations in receptor activity. Similarly, mammalian reporter assays were conducted to corroborate our findings in a higher vertebrate model system. Our current data shows that, mechanistically, the H1-H3 loop is a relevant FKBP regulatory surface for the steroid hormone receptors AR and GR, and suggests that the

H1- H3 loop may represent a novel target surface for the simultaneous inhibition of AR, GR, and PR.

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CHAPTER 1: BACKGROUND

1.1 Prostate cancer

Prostate cancer is the most common non-cutaneous, life-threatening malignancy diagnosed among American men (1, 2). According to the American Cancer Society, for the year 2024, there will be an estimated total of 299,010 new cases of prostate cancer, of which 35,250 will result in death. It arises from the prostate gland, a walnut-sized organ located just below the bladder (3, 4). Recent evidence points out that initiation and progression of prostate cancer are significantly influenced by the actions of steroid hormone receptors (SHR) (5–7). The steroid hormone receptor family is comprised of the Androgen Receptor (AR), the Glucocorticoid Receptor (GR), Estrogen Receptor (ER), Progesterone Receptor (PR) and Mineralocorticoid Receptor (MR) (8). The AR plays a significant role in the maintenance and development of prostate cancer and is activated by androgens such as testosterone and dihydrotestosterone (DHT). Therefore, initial therapeutic strategies for the treatment of prostate cancer focus on androgen deprivation to lower the levels of hormones in the body and anti-androgens that compete with hormone binding. However, resistance develops quickly leading to a more aggressive and lethal form of the disease known as castration-resistant prostate cancer (CRPC) (9–17).

Currently, de novo strategies targeting prostate cancer consider the AR BF3 surface to be important for regulating the androgen receptor signaling pathway. Additionally, GR signaling seems to be a common mechanism implicated in anti-androgen resistance and suggests the H1-H3 loop on the receptor LBD as another putative regulatory surface (18). However, more studies must be conducted to elucidate the mechanisms behind its functioning.

1.2 Androgen receptor signaling and structure

AR is responsible for male sexual differentiation and plays a pivotal role in the development and progression of prostate cancer (11, 12, 19, 20). It is located on the X chromosome at position Xq11-Xq12 and contains 8 exons which encodes for a 110 kDa protein of 919 amino acids (2757 nucleotides) (9, 14, 21–25). AR, as well as all the other members of the SHR family, share functional and structural similarities (16, 22, 24). Their structure is composed of 4 functionally distinct domains: a poorly conserved N-terminal domain (NTD), a highly conserved DNA binding domain (DBD), a hinge region, and a moderately conserved C-terminal ligand-binding domain (LBD) (Fig. 1). The NTD is located on exon 1 and contains the ligand-dependent activation function 1 (AF-1) which is required for maximal activity of the AR. The DBD contains 68 amino acids that are located on exons 2 and 3 (23). The LBD is responsible for hormone binding, coactivators recruitment, mediating homo and hetero-dimerization and ligand-dependent transcriptional activity (9, 14). It encompasses 12 alpha helices numbered from H1-H12, which help in LBD folding, and contains the ligand-dependent activation function 2 (AF-2) responsible for forming the coregulator binding site. Binding of coregulators at the AF-2 surface is influenced by another regulatory surface adjacent to it, known as the BF-3 surface (23, 26, 27) (Fig. 2).

The AR, in its inactive form, is localized in the cytoplasm as part of a complex that includes heat-shock proteins like Hsp90, 70 and 40, as well as cochaperones like the immunophilins FKBP52 and FKBP51 (16, 24) [Fig. 3]. The importance of these proteins is to prevent premature degradation of the AR, and to provide it with the needed conformation for ligand binding. The biological action of the AR is initiated upon binding

of DHT to the ligand-binding pocket, causing a conformational change that promotes the dissociation of heat shock proteins from the receptor. The activated AR then translocate into the nucleus where it binds to the androgen response element (ARE) to initiate transcription of target genes that promote growth and survival (9, 11, 16, 19, 24).

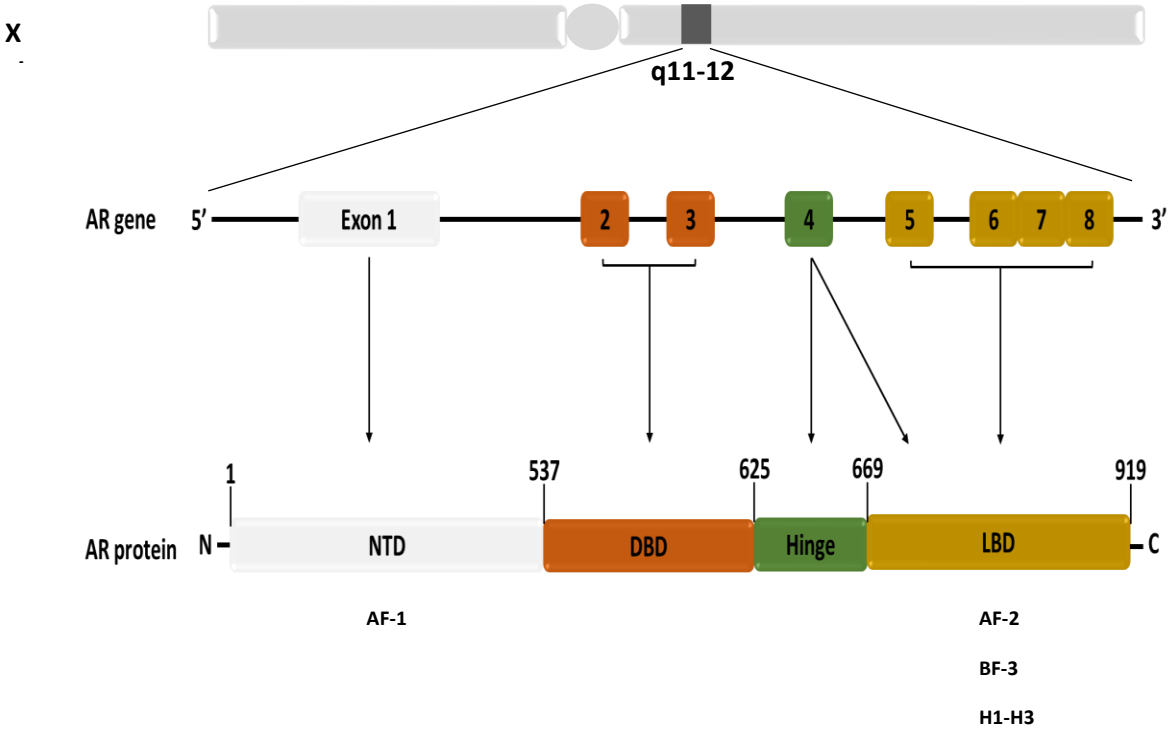

Fig. 1. Modular Structure of the Androgen Receptor. *The AR is composed of 4 functionally distinct domains: The N-terminal domain (NTD), the DNA-binding domain (DBD), a small hinge region, and the C-terminal ligand-binding domain (LBD). The NTD contains the activation function site 1 (AF-1), which is necessary for optimal transactivation of target genes. The DBD utilizes two zinc fingers to bind the major groove of DNA. The hinge region grants the receptor structural flexibility and aids in AR co-activator recruitment. The LBD contains the activation function 2 (AF-2), which is regulated by the BF-3 surface, also found in the LBD. This region also contains the H1-H3 loop, which has been proposed as a putative regulatory surface for the FKBP proteins. The LBD plays a critical role in receptor activation due to the regulation of hormone-binding.*

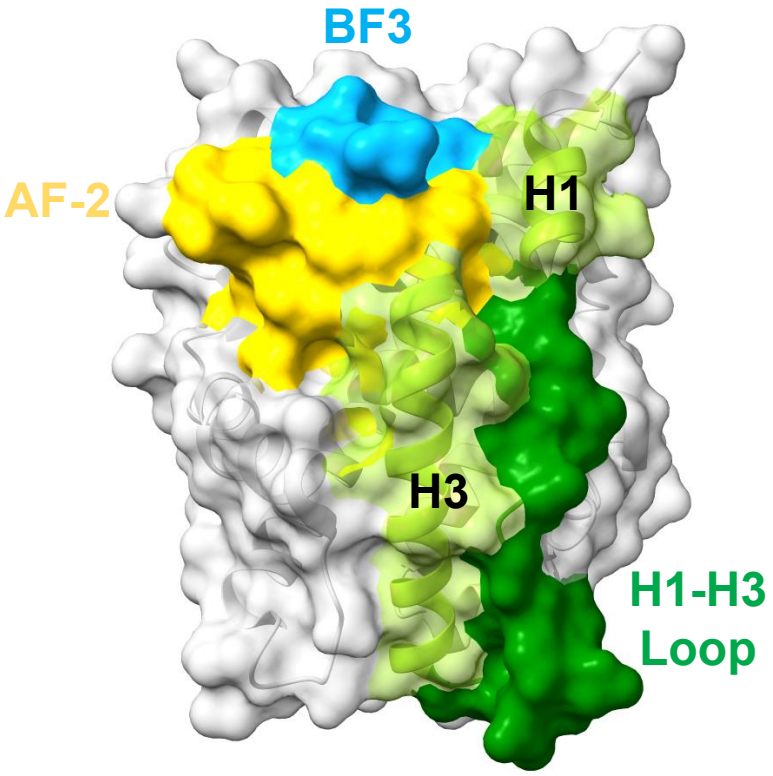

Fig. 2. Androgen receptor ligand binding domain. *The LBD of the AR is responsible for hormone binding, which induces a conformational change in the receptor, exposing a nuclear localization signal. It also regulates coactivator recruitment due to its activation function 2 (AF-2) and binding function 3 (BF3) sites. The role of the H1-H3 loop has not been determined yet, but it has been proposed to act as a regulatory surface for FKBP52 by promoting conformational changes in BF3. The figure was created using UCSF ChimeraX.*

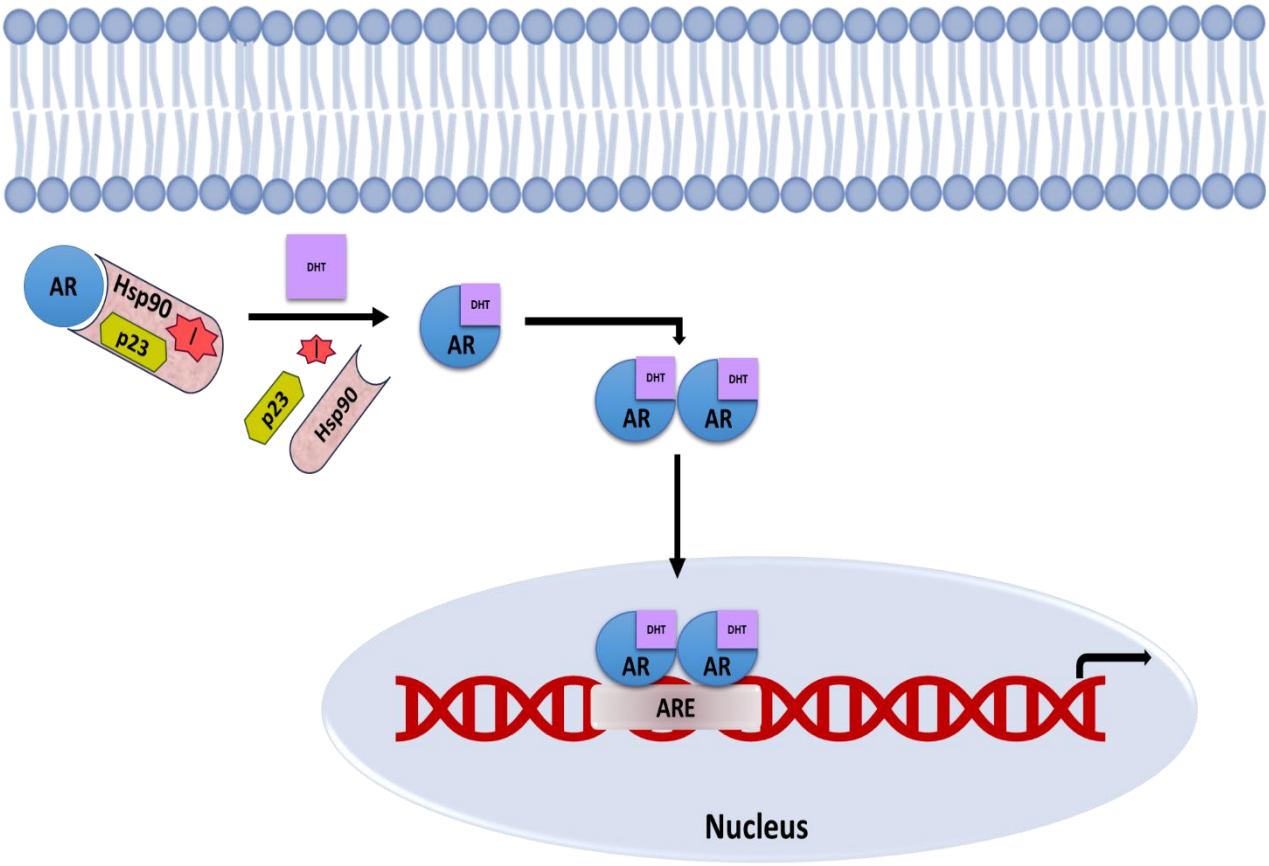

Fig. 3. Classic androgen receptor signaling. *Unliganded AR is located in the cytoplasm bound to chaperone proteins. It is only after binding to dihydrotestosterone that the receptor releases the chaperone proteins, enabling dimerization and nuclear translocation. Upon entering the nucleus, AR recognizes androgen response elements (ARE) on the DNA where it binds to and starts the recruitment of transcription co-activators to the promoter and enhancer regions of the target gene, resulting in the subsequent transcription of AR-regulated genes.*

1.3 Chaperone-mediated receptor maturation

Steroid Hormone Receptors folding, and hormone-dependent activation involves no less than twelve proteins, many of which are potential therapeutic targets for prostate cancer treatment, and at least three distinct complexes through which it cycles chronologically (Fig. 4). Early in the complex, Hsp40 binds to the receptor to later recruit Hsp70. At this moment, the receptor can move towards proteasomal degradation or continue to the intermediate complex. Once the receptor is in the intermediate complex, SGTA binds to Hsp70, which then recruits HIP and HOP in order to allow Hsp90 to bind to the complex. The final complex in which the receptor is capable of high affinity hormone binding so it can translocate into the nucleus, dimerize and bind to the response element to initiate gene transcription, includes the Hsp90 protein, the p23 cochaperone, and one of the several immunophilins like the FKBP proteins 51 and 52 (FKBP51 and FKBP52) (18, 28).

1.3.1 Early complex

As the receptor leaves the ribosomes, it begins its association with Hsp40, which recruits Hsp70 by stimulating its ATPase activity (29). This process promotes the interaction with Hsp90 through an ATP-dependent manner. At this stage, the fate of the folding cycles is determined through “protein triage”, in which the decision of whether the receptor will be allowed to move to the intermediate complex or be directed to the ubiquitin/proteasomal pathway for degradation will be made (30, 31). If the receptor is folded improperly, it will continue its way to proteasomal degradation by the tetratricopeptide repeat (TPR)-containing co-chaperone CHIP and the co-chaperone Bcl-2-associated athanogene BAG-1. CHIP binds to the TPR domain of Hsp70 or Hsp90 blocking chaperone maturation, while its U-box E3 ubiquitin ligase activity elicits ubiquitylation, targeting the

receptor for degradation. BAG-1 cooperates with CHIP by recruiting Hsp70 to the proteasome for degradation (30, 32–34).

1.3.2 Intermediate complex

The intermediate complex begins forming with the interaction of SGTA with Hsp70, which then recruits Hip through its N-terminal TPR domain. This interaction is important for preventing ADP dissociation from Hsp70, which facilitates the association of the steroid hormone receptor with Hsp90 and Hop (18). Hop is a TPR-clamp domain co-chaperone consisting of TPR1, TPR2A, and TPR2B, which coordinates the binding of Hsp90 and Hsp70 through two independent TPR domains, forming a bridge between the two chaperones that helps with the transfer of client proteins (18, 29, 34–36). It is important to note that the dissociation of Hip, Hop, and Hsp70 from the complex is essential for the progression of the chaperone cycle to the mature complex (31, 37).

1.3.3 Mature complex

Progression into the mature complex starts once Hsp90 recovers its ATPase activity, resulting in a conformational change essential for chaperone activity (34, 37). It is at this moment that Hip, Hop, and Hsp70 are released from the complex, while p23 and one of the TPR-containing immunophilins (such as FKBP52) simultaneously bind stabilizing the receptor-Hsp90 complex to which hormone can bind with high affinity (29, 31, 34, 36, 38–40). The mature complex will remain in the cytoplasm until ligand binding or the dissociation of Hsp90 leads to the recycling of the receptor. Upon ligand binding, the receptor dimerizes and translocates to the nucleus, where it binds hormone response elements (HRE) that initiate gene transcription (18, 34, 41).

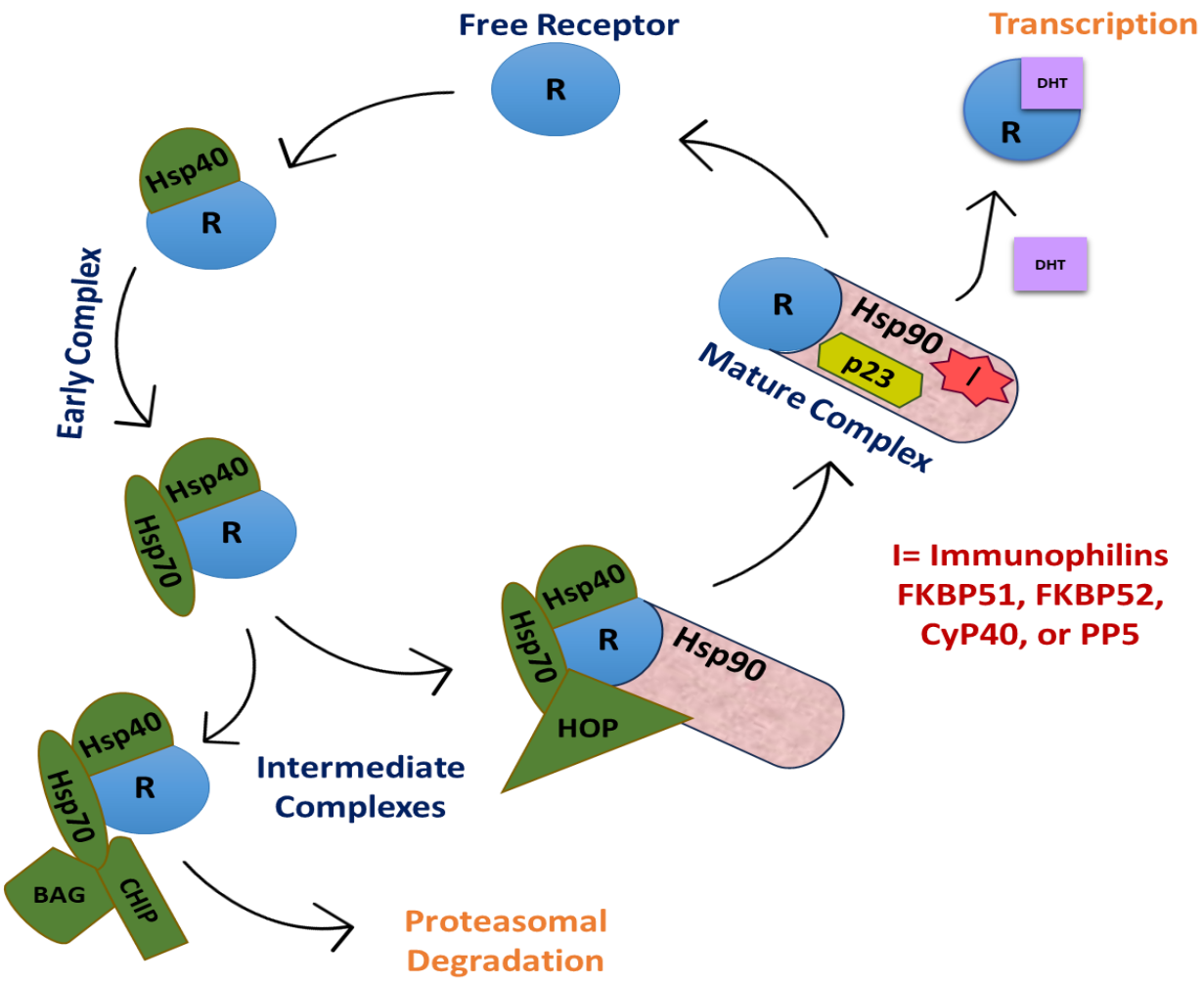

Fig. 4. Chaperone-Mediated Assembly of Steroid Hormone Receptors. The receptor, early after being translocated onto the ribosomes, begins associating with Hsp40 and later recruits Hsp70, forming the early chaperone complex. It is at this moment the receptor can either progress to an intermediate complex, where HIP and HOP bind to Hsp70 to recruit Hsp90, or the receptor can go through proteasomal degradation, in which the ribosome has been miss-folded. Therefore, if the ribosome has been misfolded the carboxyl terminus of CHIP and Bag proteins directs the receptor towards ubiquitin and proteasomal degradation pathways. Once the receptor is in the intermediate state and

has bound HIP, HOP and Hsp90, the receptor moves onto a mature complex where immunophilins FKBP52, FKBP51, CyP40 and PP5 compete for binding to Hsp90. Along with this process, p23 binds in which it stabilizes Hsp90 interactions in order to reach a high affinity binding conformation, known as the final mature complex. The mature complex will remain in the cytoplasm until hormone binding, when the complex dissociates, and the receptor is imported into the nucleus for the activation of target genes.

1.4 FKBPs structure and receptor association

FKBP proteins 51 and 52 are highly promising therapeutic targets for the disruption of mechanisms important in several endocrine-related diseases such as prostate cancer. As a result, these two proteins have become the focus on the identification and development of new drugs that target them. FKBP52 is known for being a positive regulator of AR, PR and GR activity, whereas FKBP51 has been characterized as a negative regulator of steroid hormone receptor activity, presumably through direct competition for binding of Hsp90 (18, 42–44).

FKBP51 and FKBP52 share approximately 80% sequence and structure similarity [Fig. 5 (35, 45)], they both contain: a highly conserved TPR domain that mediates binding to Hsp90, an FK1 domain which displays PPIase activity, and contains the proline-rich loop which is crucial for receptor regulation and may serve as a functionally receptor interaction surface, an FK2 domain which is structurally similar to FK1, but lacks PPIase activity. These two domains are connected by the FK linker, and a C-terminal tail that also influences Hsp90 binding. Yet, despite all these similarities, they are functionally distinct (18, 35, 42, 46, 47).

1.4.1 TPR Domain

The TPR domain is composed of tandem repeats of 34 amino acid motifs, which assume a helix-loop-helix conformation that form a concave binding pocket that mediates protein-protein interactions (18, 48, 49). When the TPR motifs have formed, they interact with the MEEVD pentapeptide sequence in the extreme C-terminus of Hsp90 (18). Outside the TPR domain, there is a conserved region of 20 amino acids termed the Charge-Y motif, which seems to play an important role in moderating Hsp90 binding (18, 35). Immunophilins FKBP51 and 52 are known to compete for binding to the MEEVD sequence in the extreme C-terminus of Hsp90 by way of their TPR domains, which has functional consequences (18, 35, 42, 50, 51).

1.4.2 FK1 Domain

The FK1 domain is located in the N-terminus of the FKBP co-chaperone proteins and contains a functional PPIase pocket that has enzymatic activity critical for regulation of steroid hormone receptor function (18, 35, 52, 53). This domain, but not the PPIase pocket, has been demonstrated to be of crucial importance for FKBP function through interactions with the steroid hormone receptor LBD (18, 35, 42, 54–56).

1.4.3 FK2 Domain

There is little known about the FK2 domain and further studies need to be conducted on this domain to determine the residues and regions that are critical for regulation of receptor function. We know that the FK2 domain is similar to the FK1 domain but lacks PPIase activity. The FK2 domain is absolutely required in order to maintain the overall size and structure of the FKBPs. Mutagenesis and chimeric proteins studies using FKBP51 and FKBP52 were conducted to show that the FKBP52 FK2 domain is critical

for full receptor potentiating ability, due to interactions between the FK2 and TPR domains (18, 35, 42, 57).

1.4.4 FK Linker

The FK domain (FK linker) contains a short amino acid long flexible hinge region that connects the FK1 domain to the FK2 domain, as well as a consensus casein kinase II (CKII) phosphorylation site (TEEED) that is present in FKBP52 but not FKBP51, and which has demonstrated to disrupt FKBP52 binding to Hsp90 (18, 35, 42). When phosphorylation occurs the FK linker region is destabilized, causing a reorientation within the linker that induces an allosteric rearrangement of the FK1 domain (18, 58, 59). The FK1 domain and the proline-rich loop play a critical role in forming an interaction site and for receptor potentiation.

1.5 Current Focus

Our lab has been able to identify which regions on FKBP52 are necessary for receptor activity and the putative FKBP52 regulatory sites on AR, and we believe this regulation is occurring on the LBD of the AR, more specifically, the BF3 surface. Previous studies have identified the BF3 surface as an AR-specific regulatory site for FKBP52, and it is likely that there is another common regulatory surface among all the regulated receptors, like the helix 1 to 3 loop, which has been proposed as a putative regulatory surface for

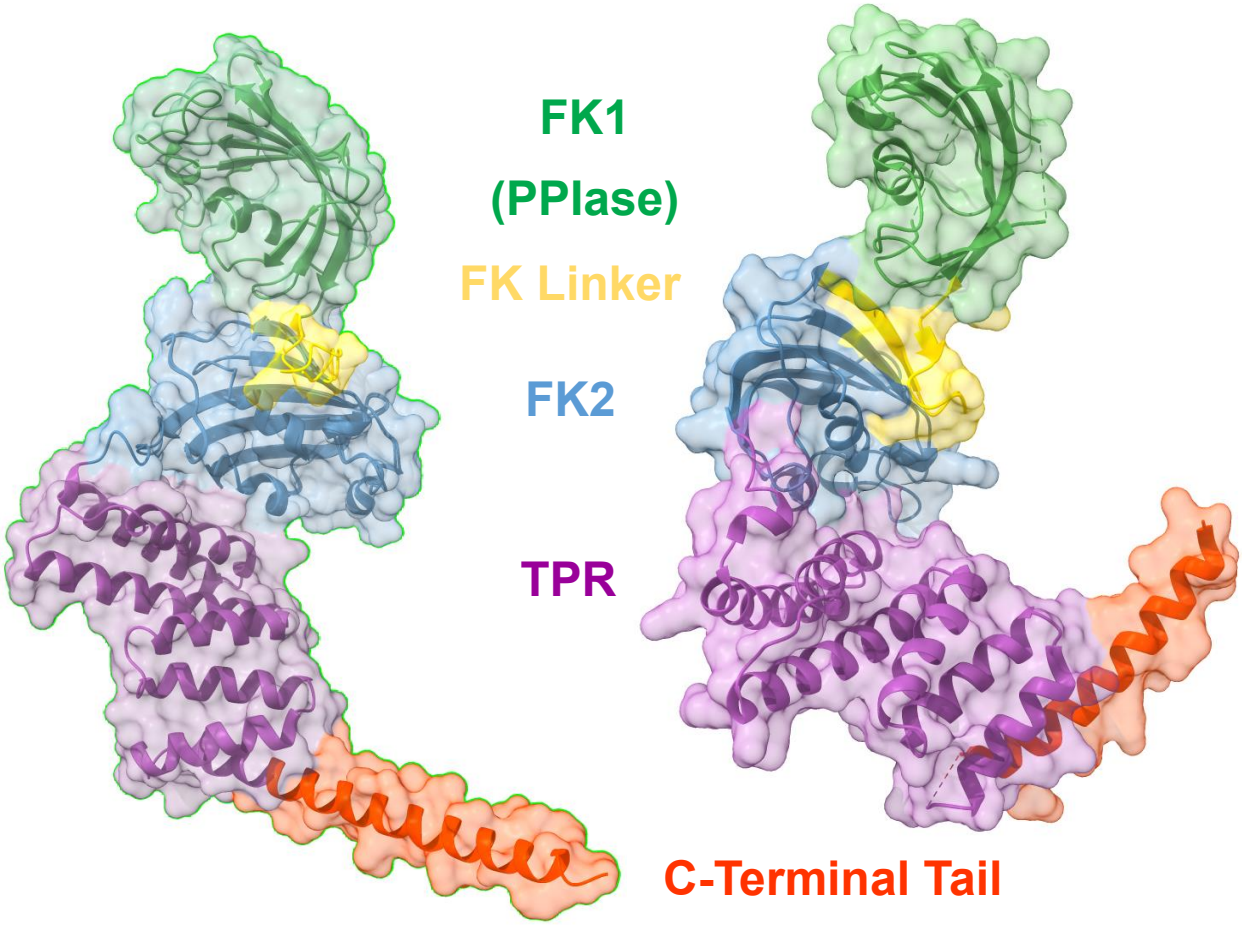

Fig. 5 FKBP51 and FKBP52 X-ray Crystallographic Structures and Functional Domains*. The tree-dimensional structure of human FKBP51 (PDB# 1KT0) and an overlap of two partial structures encompassing the full length human FKBP52 (PDB# 1Q1C and 1P5Q) are represented in the figure, and their functional domains are illustrated. The C-terminal TPR domain mediates Hsp90 binding. FK1 and FK2 are similar to each other, but FK2 lacks PPIase activity. The proline-rich loop overhanging the PPIase pocket is crucial for receptor regulation and may serve as an important interaction surface. The figure, including the composite of the two partial FKBP52 structures, was created using UCSF ChimeraX.*

the FKBP proteins in GR. GR is known to be more sensitive to FKBP52 regulation as compared to AR, despite their common structural organization (Fig. 6) (54, 56).

The amino acid sequence within the helix 1 to helix 3 loop in the receptor ligand binding domain of the GR is fully conserved between human and the receptors of several other species, but it differentiates from the guinea pig GR by 5 amino acid substitutions (Fig. 7). Furthermore, in guinea pig GR, decreased glucocorticoid sensitivity has been attributed to the combined effect of these 5 nonconservative residue changes, which might disrupt a contact domain for the FKBP proteins (60–64).

Previous studies have identified the H1-H3 loop on the GR LBD as an important target for receptor regulation by the FKBP co-chaperones (65). Cluning et al looked at the role of the nonconservative mutations within the H1-H3 loop on the regulation of GR activity by the FKBP cochaperones, demonstrating that this loop is not a direct interaction site for FKBP52. However, these mutations affected FKBP52-mediated receptor activities. Thus, the H1-3 loop may be acting as a regulatory surface that promotes conformational changes within the receptor Hsp90 complex.

Thus, we were interested in exploring the question of whether or not the H1-3 loop could serve as a common FKBP regulatory surface for all FKBP52-regulated receptors (AR, PR, and GR)?

The overarching goal of this research project was to demonstrate the functional relevance of the H1-H3 loop with the objective of identifying the regulatory surface for FKBP51 and FKBP52 co-chaperones within the context of Hsp90 chaperone complex.

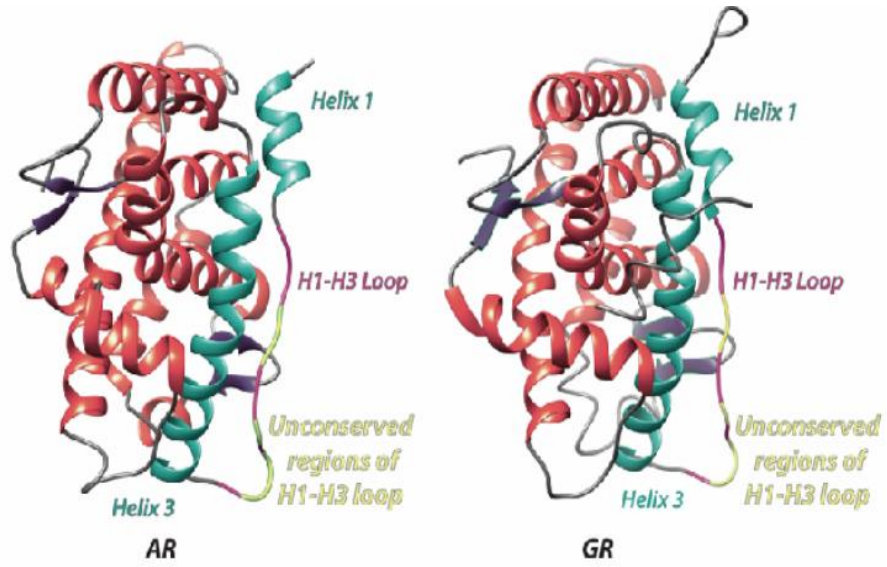

Fig. 6. 3-D Structural Comparison of hSHR AR and GR LBD. *The H1-H3 loop in hAR, hGR, and gpGR (not shown) is mostly conserved, except were indicated (yellow). While gpGR LBD has not yet been crystalized, previous studies have shown varying FKBP52-regulated activity between the SHRs which is likely attributed to the un-conserved regions within the H1-H3 loop may serve as a common site for FKBP52 regulation of SHRs AR, GR, and PR.*

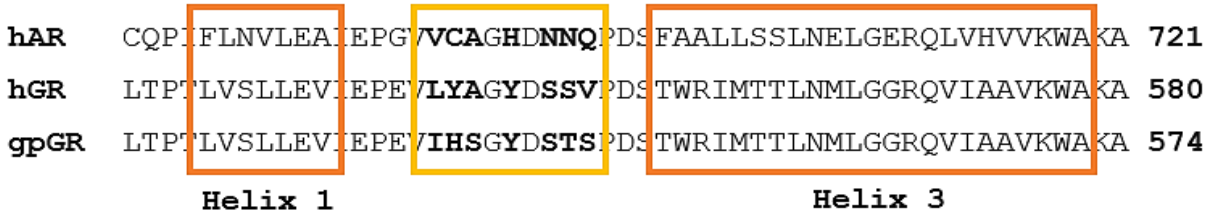

Fig. 7. Nonconservative mutations within the H1-H3 loop of the Androgen and Glucocorticoid receptors Ligand Binding Domain. *The sequences from the helix 1 to helix 3 within the ligand binding domain of the human Androgen Receptor (AAA51729.1), human Glucocorticoid Receptor (NP_001018087.1), and guinea pig Glucocorticoid Receptor (NP_001166458.1) were aligned using the Clustal Omega program. The amino acids constituting the two helixes are marked by an orange box and the nonconservative residues with the yellow box and highlighted in black.*

1.6 HYPOTHESIS AND SPECIFIC AIMS

The H1-H3 loop is a common regulatory surface for FKBPs for AR, GR, and PR, and differences within this surface confer GR hypersensitivity to FKBP52.

1.6.1 Specific Aim 1: Site-directed mutagenesis to generate a class of mutans that will make the H1-H3 loop of hAR similar to the loop of the gpGR and assess their effects on FKBP regulation through yeast-based assays.

1.6.2 Specific Aim 2: Site-directed mutagenesis to generate a class of mutans that will make the H1-H3 loop of hAR similar to the loop of the hGR and assess their effects on FKBP regulation through yeast-based assays.

1.6.3 Specific Aim 3: Corroborate the yeast-based findings and assess mutants of interest for functional effects on FKBP regulation in human cellular model systems.

CHAPTER 2: SITE-DIRECTED MUTAGENESIS TO GENERATE A CLASS OF MUTANTS THAT WILL MAKE THE HELIX 1 TO HELIX 3 LOOP OF THE WILD-TYPE HUMAN ANDROGEN RECEPTOR LIGAND BINDING DOMAIN SIMILAR TO THE LOOP OF THE GUINEA PIG GLUCOCORTICOID RECEPTOR AND ASSESS THEIR EFFECTS ON FKBP REGULATION THROUGH YEAST-BASED ASSAYS.

2.1 Rationale

The amino acid sequence within the helix 1 to 3 loop (H1-H3) in the receptor ligand binding domain (LBD) is conserved among the steroid hormone receptor superfamily of different species. Previous studies have associated 5 amino acid substitutions within the loop of the guinea pig Glucorticoid Receptor (gpGR) with reduced glucocorticoid sensitivity. Additionally, Cluning et al used these nonconservative mutations to elucidate their functional effects on FKBP-mediated receptor regulation, showing that changes to this loop alter receptor activity by disrupting a contact domain for the FKBP co-chaperones promoting conformational changes that affect hormone binding (65).

Based on these findings, we aimed to demonstrate the functional relevance of the H1-H3 loop on the human Androgen Receptor (hAR). Taking advantage of the differences between AR and GR, we conducted site-directed mutagenesis to generate a class of mutants that made the hAR similar to the loop on the gpGR and assessed the role and relevance of those amino acid substitutions on receptor activity through yeast-based assays.

2.2 Materials and Methods

2.2.1 Q5 Site Directed Mutagenesis

The hAR-LBD DNA from the plasmid *pBluescript SKII-* was used as a template to generate the gpGR-LBD H1-H3 loop mutant constructs. Oligonucleotide primers were designed online with the help of the NEBaseChanger tool from New Englad BioLabs Inc. and synthesized from IDT Inc. Mutagenesis was performed with the Q5 Site-Directed Mutagenesis Kit form New England BioLabs according to the manufacturer's instructions. Briefly, an exponential amplification (PCR) using the previously designed primers, and a

master mix formulation of Q5 Hot Star High-Fidelity DNA Polymerase were assembled in a PCR tube and transferred to a thermocycler under the following conditions: initial denaturation at 98 degrees Celsius for 30 seconds, 25 cycles at 65 degrees Celsius for 2 minutes, and a final extension at 72 degrees Celsius for 2 minutes. After the PCR was done, the samples were treated with a Kinase, Ligase and DpnI (KLD) mixture for 5 minutes. Finally, the samples were transformed into NEB 5-alpha Competent E. coli cells and left overnight at 37 degrees Celsius. Once mutagenesis was confirmed by sequencing, the mutated region from the hAR-LBD DNA was excised from *pBluescript SKII-* with the restriction enzymes HindIII and XhoI and ligated into the p425GPD cloning vector containing the full-length AR using the same restriction enzymes. Correct orientation of the inserted fragments was checked with KpnI digests.

2.2.2 Yeast Strains

The wild type W3031b (MATa leu2–112 ura3–1 trp1–1 his3–11, 15 ade2–1 can1–100 GAL SUC2) was used as background to originate all *S. cerevisiae* strains. Plasmids constitutively expressing the lacZ gene as a hormone-inducible reporter plasmid, FKBP52, FKBP51 or empty vector, and either WT-AR or the AR-LBD mutants were introduced into wild type yeast strain using a lithium acetate/polyethylene glycol protocol, as described previously (66). Briefly, W3031b cells were grown overnight in YEPD liquid medium at 30°C. After 16-18 hrs., centrifuge the overnight growth at 2000 × g for 3 min, and wash with 1 ml of 1X TE/LiAc twice. Mix 50 µL of washed yeast cells, 5 µL denatured salmon sperm DNA, 3 µL of the appropriate plasmid, and 300 µL PEG/TE/LiAc in a 1.5 mL microcentrifuge tube, and incubate at 30°C for 30 min followed by 12 min incubation at 42°C. Then, centrifuge the cells for 30 sec at maximum speed and resuspend in 100

µL sterile deionized, distilled water (ddH_2O). Finally, spread the entire cell suspension on an agar plate containing synthetic complete media lacking the appropriate amino acids to select for plasmid uptake and retention, and incubate at 30°C for 3-4 days. Procedure was repeated for each plasmid to be co-transformed. 3 independent transformants of each generated strain were compared for consistent properties.

2.2.3 Yeast Reporter Assays

Functional assays were conducted as described previously (66). Briefly, all hormone-dependent reporter assays were performed in the yeast strains described above. Cells were grown overnight in synthetic complete media lacking the amino acids leucine, uracil and tryptophan (SC-LUW) at 30°C in a shaking water bath. The next day, cultures were diluted to an optical density of 0.08 at 600 nm ($O.D._{600}$) and incubated in a shaking water bath at 30°C to an $O.D_{600}$ of 0.1 (log phase). Growth was monitored by spectrophotometry every 30 minutes until the culture reached the desired absorbance. 100 µL of yeast cells were plated in a 96-well plate and treated with different DHT concentrations for hormonal induction. The plate was then incubated at 30°C for 2 hrs. after which, 100 µL of Tropix Gal-Screen in Buffer B (Applied Biosystems) were added to each well and the plate was incubated at room temperature for an additional 2 hrs. The plate was read in order to measure relative light units (RLU) using a Synergy 2 Multi-Mode Microplate Reader (BioTek). The rate of increase in β-galactosidase expression of light units was plotted against increases in DHT concentrations.

2.2.4 Western Analysis

Yeast cells were grown as for the reporter assay and lysed with a lysis buffer containing protease inhibitors. Whole-cell protein was electrophoresed through a 10% SDS-PAGE

gel and transferred for 1 hr. to a nitrocellulose membrane at 110 V at 4°C. The membrane was incubated in blocking buffer (1× Tris-buffered saline/0.02% Tween 20 [TBS-T] [pH 7.6], 3% skim milk) for 1 hour at room temperature and then incubated with primary antibody diluted in blocking buffer for 2 hours at room temperature. The following dilutions of primary antibodies were used: AR 441 (Santa Cruz Biotechnology) at 1/500, FKBP52 (Hi52c) at 1/5000, and FKBP51 (Hi51d) at 1/5000. As an internal loading control for yeast protein, the anti-actin antibody (Abcam) at 1/1000 will be used. The membrane was washed with blocking buffer (5 times for 5 minutes) and then incubated with the appropriate secondary antibody (goat antimouse) diluted 1/10 000 in blocking buffer for 1 hour at room temperature. Finally, the membrane was washed (5 times for 5 minutes) in 1× TBS-T, incubated with Immun-Star™ AP substrate (BioRad) and exposed on CL-X Posure™ film (Thermo Scientific) for detection of antibodies.

2.2.5 Statistical Analysis

The descriptive statistics were calculated, and the graphs plotted using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad. Statistical significance was determined by Two-way ANOVA followed by Dunnett's multiple comparisons test.

2.3 Results

We conducted site-directed mutagenesis to generate DNA constructs with the specific residue changes that differentiate the helix 1 to helix 3 loop of the hAR from the gpGR, individually or in combination to examine their effect on receptor potentiation by the FKBP co-chaperones (Fig. 8).

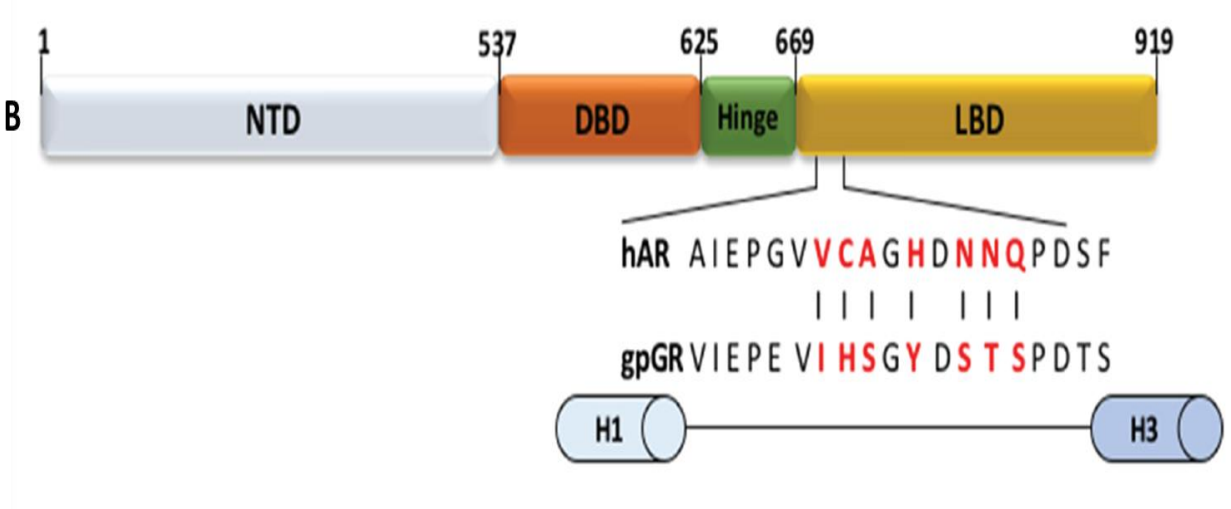

Fig. 8. Representation of the AR domain structure, with indications of the specific mutations within the H1-H3 loop of the LBD. Representation of the AR domain structure showing the residue changes done to the H1-H3 loop of the hAR LBD to make it look like the loop from the gpGR.

S. cerevisiae reporter strains were generated by transformation with a *lacZ* reporter plasmid, an expression plasmid for FKBP52, FKBP51, and an expression plasmid for wild-type or mutant hAR. Functional effects were assessed by yeast-based reporter assays (Fig. 9).

The mean reporter expression of three independent experiments for the WT-AR in the presence of FKBP51 (WT-AR+FKBP51), FKBP52 (WT-AR+FKBP52) or empty vector (WT-AR+Empty Vector) is shown below (Fig. 10). As expected, in the presence of FKBP52, there is a distinct shift in the hormone response curve with a DHT EC50 (half maximal effective concentration) of ~9-fold less than the FKBP51 and empty vector strains, which only differ between each other by 1-fold. The curves shown in Fig. 10 will be used as the baseline to compare the effect of different mutants on receptor regulation by the FKBP proteins.

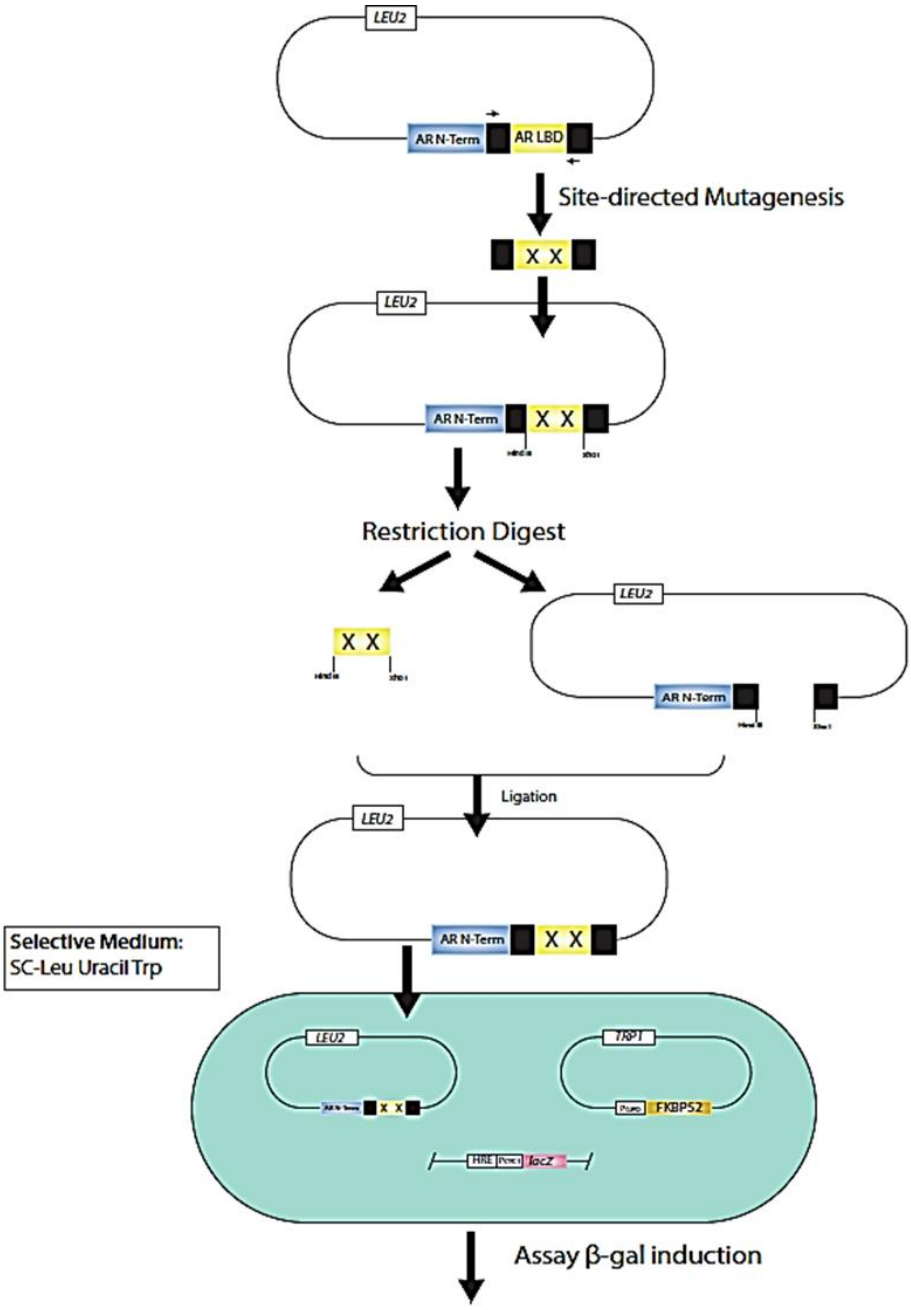

Fig. 9. Schematic diagram for Q5 site-directed mutagenesis and yeast functional assays. *The figure represents the different steps involved in the generation of DNA constructs containing the amino acid substitutions within the H1-H3 loop of the hAR to make it similar to the hGR and the gpGR, and the yeast reporter strain transformation used to assess functional effects (Figure produced by Idalys Ramirez).*

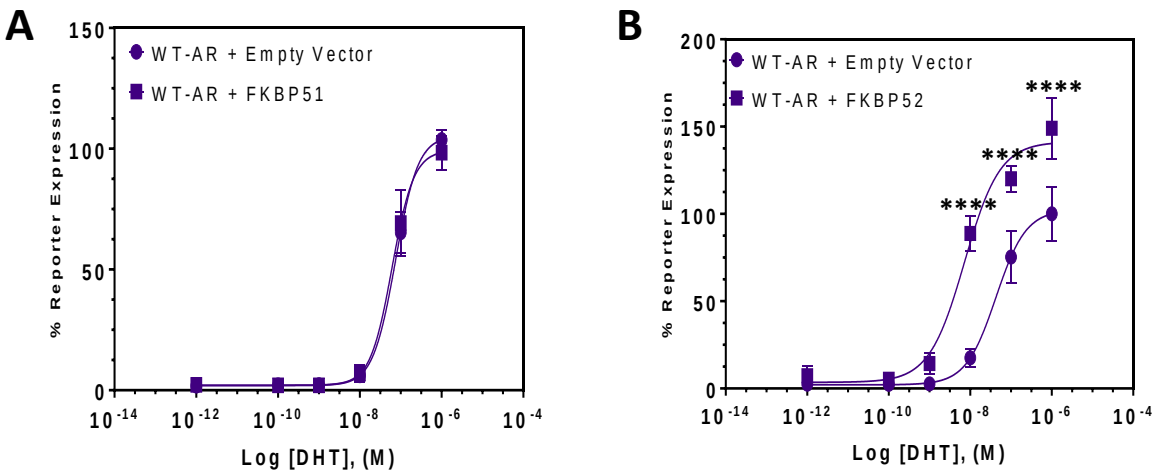

Fig. 10 DHT hormone-dose response curves for WT-AR in the presence of FKBP51, FKBP52 or empty vector. *The figure represents the mean reporter expression of three independent experiments for the WT-AR in the presence of FKBP51 (WT-AR+FKBP51), FKBP52 (WT-AR+FKBP52) or empty vector (WT-AR+Empty Vector).*

To determine whether the hAR-gpGR mutants affected FKBP-mediated receptor function, reporter gene expression was measured by dosing yeast cultures with increasing concentrations of DHT to generate full hormone dose-response curves for the WT-AR strain, mutant strains, and vector control strain (Fig. 11).

Figure 11 shows that the hAR-gpGR mutants appear to be upregulated even in the absence of any of the cochaperones FKBP51 and FKBP52. However, when in the presence of 51 and 52, receptor activity is downregulated (except for Fig. 11 D and I).

By introducing the Valine to Isoleucine (V/I) mutation in the full-size androgen receptor, we observed that the receptor activity of the mutant is increased by 200% at the highest concentration of DHT, as compared to the activity of the WT-AR. This effect is seen in the absence of either of the cochaperones FKBP52 and FKBP51 (Fig. 11 A and B). However,

when exposed to the presence of the proteins 51 or 52, the reporter expression of the receptor gets downregulated.

Similarly to the V/I mutant, by changing a Cysteine to a Histidine (C/H), the activity of the receptor is generally increased even in the absence of the protein FKBP51 (Fig. 11 C), with a higher maximal activity of 250% more than WT-AR. However, when exposed to FKBP51, the reporter expression gets downregulated by 150%. In contrast, when the mutant is exposed to the presence of FKBP52 (Fig. 11 D), we see an increment in receptor activity of about 150%, with a significant shift to the left. This means that the mutant not only increases the receptor activity, but it also makes the receptor more sensitive to the presence of the cochaperone.

The amino acid substitution of an Alanine to a Serine (A/S) increases the receptor activity by 500% in the absence of co-chaperone protein FKBP51 (Fig. 11 E) as compared to the WT-AR. However, when exposed to FKBP51, the reporter expression gets downregulated by 300%. In Fig. 11 F, we observe an increment in reporter expression of about 50% in the absence of the cochaperone FKBP52, as compared to WT-AR. When the mutant is exposed to the presence of FKBP52, receptor activity decreases approximately 100% (compared to empty vector).

In Fig. 11 G, we can observe that by introducing the Asparagine to Threonine (N/T) mutation to the WT-AR, receptor activity is increased by 500% in the absence of FKBP51 (as compared to WT-AR). However, when exposed to the co-chaperone, the same activity is downregulated by approximately 200% (when compared to N/T + Empty Vector). In contrast, in the absence of FKBP52 (Fig. 11 H), receptor activity is similar to that of WT-

AR + FKBP52. Moreover, when the co-chaperone 52 is present, a small decrease in activity can be seen in the mutant.

Fig. 11 I and J show the result in reporter expression after introducing the amino acid substitution of a Glutamine for a Serine (Q/S). In I, it can be observed an increase in reporter activity of approximately 500% after the mutation, as compared to the WT-AR. However, this change in activity seems to be independent of FKBP51. Similarly, in J, it can be observed an increment in activity of approximately 100% in the absence of FKBP52. Subsequently, when FKBP52 is added, the reporter activity is decreased to the same amount as WT-AR + FKBP52.

The mutant combination NQ/TS shows the same effect as seen with the individual mutants N/T (Fig. 11 G and H) and Q/S (Fig. 11 I and J), where reporter activity is increased in the absence of either of the co-chaperones FKBP51 (Fig. 11 K) or FKBP52 (Fig. 11 L), but when they are introduced, the activity is negatively regulated. However, this effect is more significant in the presence of 51 (150% less than NQ/TS + Empty Vector) than with 52 (50% less).

The mutant VCANQ/IHSTS combines all the different individual mutants previously discussed in figures A through J. As shown with the individual mutants, the combination increases the reporter activity in the absence of either of the co-chaperones 51 or 52, and when exposed to them, the receptor activity is downregulated. Also, in the presence of FKBP52, it can be observed a shift to the left in the dose-response curve, which suggests that the amino acid substitutions to the H1-H3 loop made the receptor more sensitive to the FKBP52 regulation, as well as increasing the activity of the receptor when exposed to higher concentrations of DHT. As seen with the other mutants, amino acid substitutions

within the H1-H3 loop seem to make the receptor more sensitive to the presence of the cochaperone FKBP52.

The mutant combination VCA/IHS seems to have made the receptor lose its dose-response activity (figure not shown). This suggests that the protein can be misconformated.

After conducting the dose-response assays, a western blot analysis of all the mutants was performed to validate the co-expression of AR and the FKBP co-chaperones (Fig. 12). In the figure, it is shown that the protein lysates obtained from the yeast assays confirm the presence of the proteins of interest. Furthermore, in the right figure, it can be seen a band at a different protein size than expected, which corroborates the idea that the protein lost receptor conformation.

As seen in the previous work by Cluning et al with GR, we also observed that amino acid substitutions within the H1-H3 loop of the hAR to make it more like gpGR impact receptor activity, as all the mutations enhanced receptor activity in the absence of FKBP51 and FKBP52. More interestingly, both FKBP51 and FKBP52 appear to negatively regulate this enhanced receptor activity in the majority of the mutants. These results combined strongly suggest that the H1-H3 acts as a relevant FKBP regulatory surface for receptor function given that changes in this loop alter the receptor's response to FKBP regulation. As a reminder, FKBP51 does not regulate wild type hAR whereas FKBP52 is a positive regulator of wild type hAR. These data also suggest that this loop is important not only for GR regulation but also for AR. However, more experiments are needed to confirm the data.

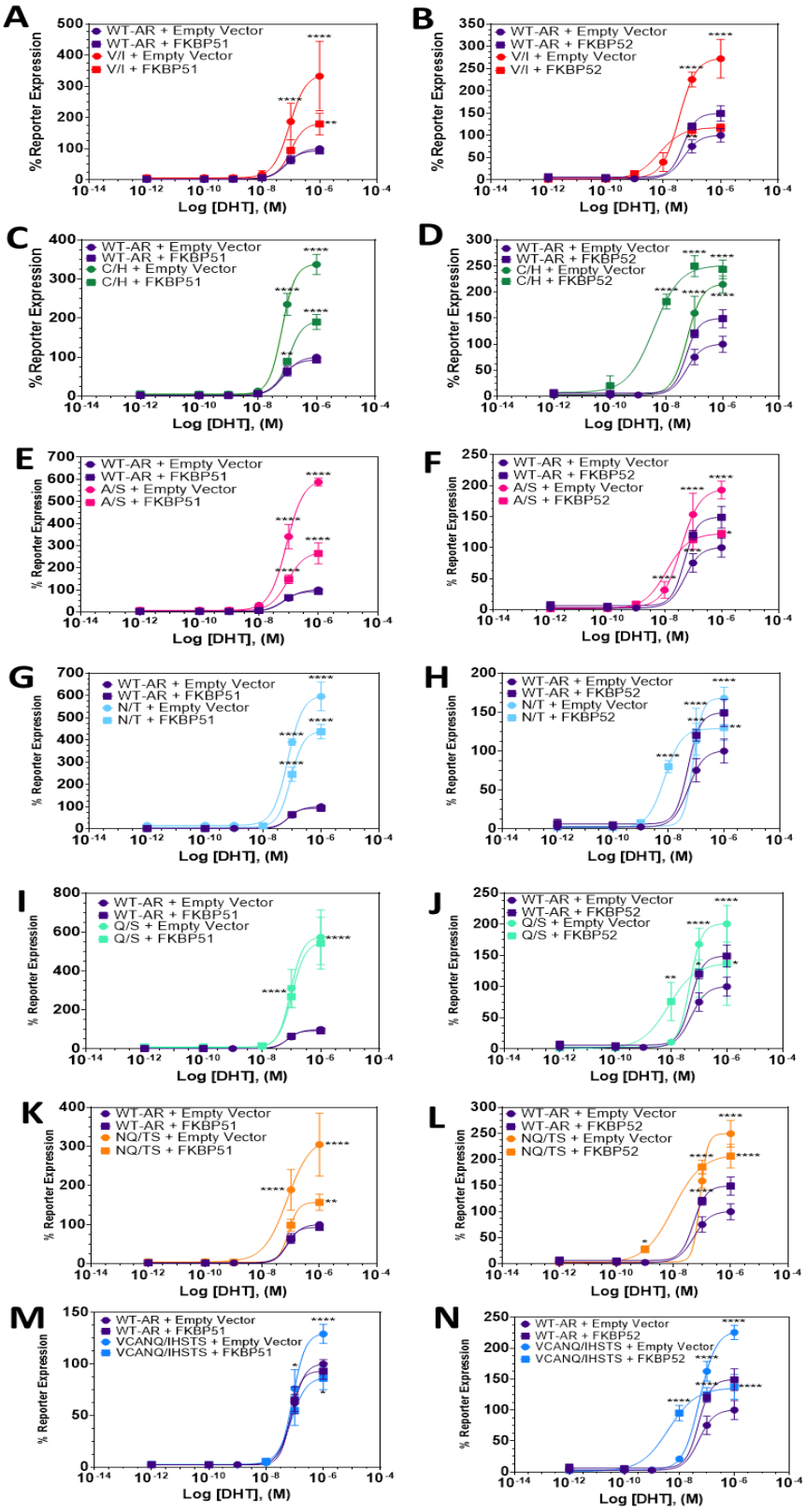

Fig. 11. hAR to gpGR Mutants in the absence and presence of FKBP51 or FKBP52

Fig.11. hAR to gpGR Mutants in the absence and presence of FKBP51 or FKBP52

(continuation). *The graphs above represent the mean reporter expression of three independent experiments for WT-AR and mutant hAR in the presence of FKBP52 (+FKBP52), FKBP51 (+FKBP51) or absence (+Empty Vector) of chaperone. Yeast S. cerevisiae were transformed with a lacZ reporter plasmid, an expression plasmid for FKBP51 or FKBP52, and an expression plasmid for wild-type hAR or the hAR to gpGR mutant and dosed with increasing concentrations of DHT. Statistical analysis was done using a 2-way ANOVA with Dunette's multiple comparisons test of normalized reporter expression rate of wild-type AR or mutant in the presence of either of co-chaperons FKBP51 or 52, against that of wild-type AR + Empty Vector.* **********, *P ≤ 0.0001;* *********, *P ≤ 0.0005;* ******* *P ≤ 0.005;* ******* *P ≤ 0.05.*

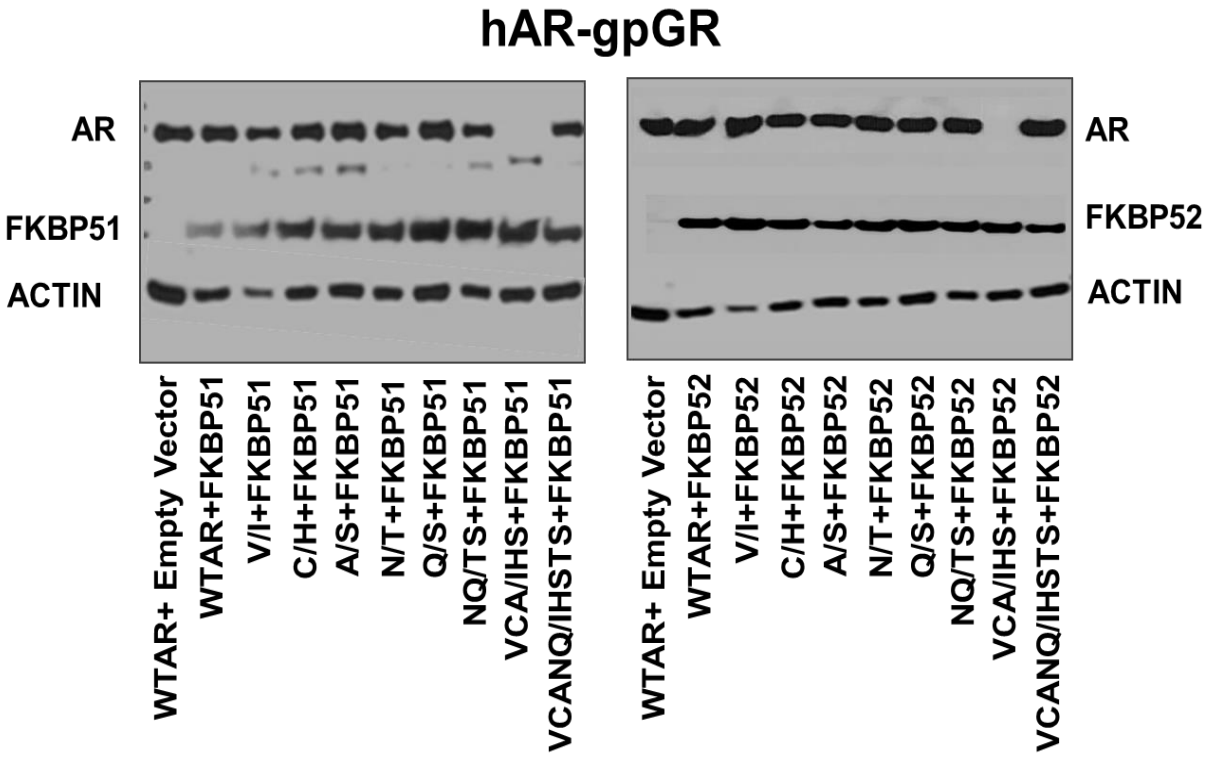

Fig. 12. Validation of the co-expression of proteins of interest. *To validate the co-expression of AR and the FKBP cochaperones 51 and 52 in our assays, we performed a Western blot analysis targeting the proteins of interest. Yeast cells were grown in selective media lacking the amino acids Leucine, Uracil and Tryptophan for 18 hrs. approximately, and lysed subsequently. Protein lysates were electrophoresed and transfer to a nitrocellulose membrane. Finally, the membrane was incubated with antibodies targeting AR, FKBP52 and FKBP51. As shown, all lysates tested positive for the presence of the proteins of interest.*

CHAPTER 3: SITE-DIRECTED MUTAGENESIS TO GENERATE A CLASS OF MUTANTS THAT WILL MAKE THE HELIX1 TO HELIX3 LOOP ON THE WILD-TYPE HUMAN ANDROGEN RECEPTOR LIGAND BINDING DOMAIN SIMILAR TO THE LOOP OF THE HUMAN GLUCOCORTICOID RECEPTOR AND ASSESS THEIR EFFECTS ON FKBP REGULATION THROUGH YEAST-BASED ASSAYS.

3.1 Rationale

Our previous studies (Chapter 2) demonstrated that changes to the helix 1 to helix 3 (H1-H3) loop on the human androgen receptor (hAR) ligand binding domain (LBD) to make it similar to the loop of the guinea pig glucocorticoid receptor (gpGR) affect FKBP-mediated receptor regulation. Thus, we hypothesized that the H1-H3 loop is a common FKBP regulatory surface for AR and GR, and differences within this surface confer GR hypersensitivity to FKBP52. Taking advantage of the distinct GR hypersensitivity to FKBP52, a class of mutants was generated through site-directed mutagenesis to make the H1-H3 loop on the hAR-LBD look like the hGR loop (Fig.13) and assessed the effects of those changes on receptor activity through yeast-based assays.

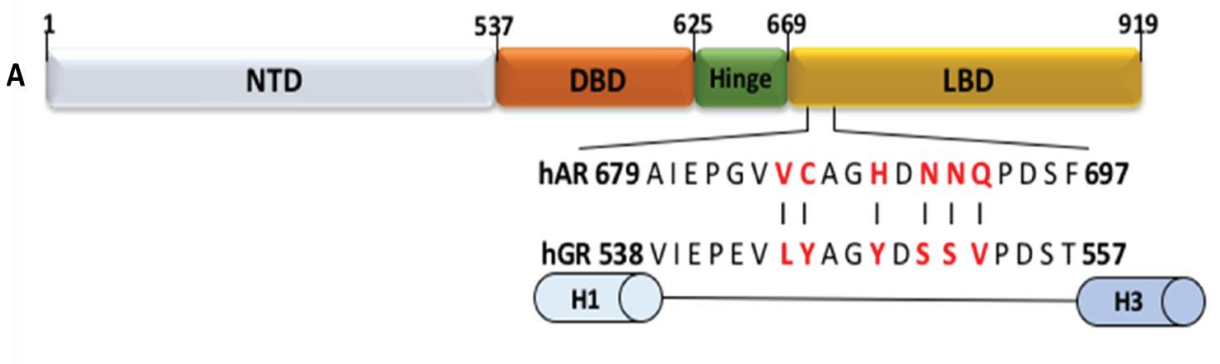

Fig. 13 hAR-hGR Mutants. Representation of the AR domain structure, with indications of the specific mutations within the H1-H3 loop of the LBD. *Representation of the AR domain structure showing the residue changes done to the H1-H3 loop of the hAR LBD to make it look like the loop from the hGR.*

3.2 Materials and Methods

3.2.1 Site Directed Mutagenesis

The hAR-LBD DNA from the plasmid *pBluescript SKII-* was used as a template to generate the hGR-LBD H1-H3 loop mutant constructs. Oligonucleotide primers were designed online with the help of the NEBaseChanger tool from New Englad BioLabs Inc. and synthesized from IDT Inc. Mutagenesis was performed with the Q5 Site-Directed Mutagenesis Kit form New England BioLabs according to the manufacturer's instructions. Briefly, an exponential amplification (PCR) using the previously designed primers, and a master mix formulation of Q5 Hot Star High-Fidelity DNA Polymerase were assembled in a PCR tube and transferred to a thermocycler under the following conditions: initial denaturation at 98 degrees Celsius for 30 seconds, 25 cycles at 65 degrees Celsius for 2 minutes, and a final extension at 72 degrees Celsius for 2 minutes. After the PCR was done, the samples were treated with a Kinase, Ligase and DpnI (KLD) mixture for 5 minutes. Finally, the samples were transformed into NEB 5-alpha Competent E. coli cells and left overnight at 37 degrees Celsius. Once mutagenesis was confirmed by sequencing, the mutated region from the hAR-LBD DNA was excised from *pBluescript SKII-* with the restriction enzymes HindIII and XhoI and ligated into the p425GPD cloning vector containing the full-length AR using the same restriction enzymes. Correct orientation of the inserted fragments was checked with KpnI digests.

3.2.2 Yeast Strains

The wild type W3031b (MATa leu2–112 ura3–1 trp1–1 his3–11, 15 ade2–1 can1–100 GAL SUC2) was used as background to originate all *S. cerevisiae* strains. Plasmids constitutively expressing the lacZ gene as a hormone-inducible reporter plasmid,

FKBP52, FKBP51 or empty vector, and either WT-AR or the AR-LBD mutants were introduced into wild type yeast strain using a lithium acetate/polyethylene glycol protocol, as described previously (66). Briefly, W3031b cells were grown overnight in YEPD liquid medium at 30°C. After 16-18 hrs., centrifuge the overnight growth at 2000 × g for 3 min, and wash with 1 ml of 1X TE/LiAc twice. Mix 50 µL of washed yeast cells, 5 µL denatured salmon sperm DNA, 3 µL of the appropriate plasmid, and 300 µL PEG/TE/LiAc in a 1.5 mL microcentrifuge tube, and incubate at 30°C for 30 min followed by 12 min incubation at 42°C. Then, centrifuge the cells for 30 sec at maximum speed and resuspend in 100 µL sterile deionized, distilled water (ddH_2O). Finally, spread the entire cell suspension on an agar plate containing synthetic complete media lacking the appropriate amino acids to select for plasmid uptake and retention, and incubate at 30°C for 3-4 days. Procedure was repeated for each plasmid to be co-transformed. 3 independent transformants of each generated strain were compared for consistent properties.

3.2.3 Yeast Reporter Assays

Functional assays were conducted as described previously (66). Briefly, all hormone-dependent reporter assays were performed in the yeast strains described above. Cells were grown overnight in synthetic complete media lacking the amino acids leucine, uracil and tryptophan (SC-LUW) at 30°C in a shaking water bath. The next day, cultures were diluted to an optical density of 0.08 at 600 nm ($O.D._{600}$) and incubated in a shaking water bath at 30°C to an $O.D_{600}$ of 0.1 (log phase). Growth was monitored by spectrophotometry every 30 minutes until the culture reached the desired absorbance. 100 µL of yeast cells were plated in a 96-well plate and treated with different DHT concentrations for hormonal induction. The plate was then incubated at 30°C for 2 hrs. after which, 100 µL of Tropix

Gal-Screen in Buffer B (Applied Biosystems) were added to each well and the plate was incubated at room temperature for an additional 2 hrs. The plate was read in order to measure relative light units (RLU) using a Synergy 2 Multi-Mode Microplate Reader (BioTek). The rate of increase in β-galactosidase expression of light units was plotted against increases in DHT concentrations.

3.2.4 Western Analysis

Yeast cells were grown as for the reporter assay and lysed with a lysis buffer containing protease inhibitors. Whole-cell protein lysates were electrophoresed through a 10% SDS-PAGE gel and transferred for 1 hr. to a nitrocellulose membrane at 110 V at 4°C. The membrane was incubated in blocking buffer (1× Tris-buffered saline/0.02% Tween 20 [TBS-T] [pH 7.6], 3% skim milk) for 1 hour at room temperature and then incubated with primary antibody diluted in blocking buffer for 2 hours at room temperature. The following dilutions of primary antibodies were used: AR (Santa Cruz Biotechnology) at 1/500, and FKBP52 (Hi52c) at 1/5000 and FKBP51 (Hi51d). As an internal loading control for yeast protein, the anti-actin antibody (Abcam) at 1/1000 was used. The membrane was washed with blocking buffer (5 times for 5 minutes) and then incubated with the appropriate secondary antibody (goat antimouse or goat antirabbit) diluted 1/10 000 in blocking buffer for 1 hour at room temperature. Finally, the membrane was washed (5 times for 5 minutes) in 1× TBS-T, incubated with Immun-Star™ AP substrate (BioRad) and exposed on CL-X Posure™ film (Thermo Scientific) for detection of antibodies.

3.2.5 Statistical Analysis

The descriptive statistics were calculated, and the graphs plotted using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA,

www.graphpad. Statistical significance was determined by Two-way ANOVA followed by Dunnett's multiple comparisons test.

3.3 Results

Based on the findings from our previous studies (Chapter 2), we conducted site-directed mutagenesis to generate DNA constructs with the specific residue changes that differentiate the helix 1 to helix 3 loop of the hAR from the hGR, individually or in combination to examine their effect on FKBP-mediated receptor potentiation (Fig. 14). For this purpose, *S. cerevisiae* reporter strains were generated as described on page 35 (3.2.2 Yeast Strains), and functional effects were assessed by yeast-based reporter assays.

In Fig. 14 A, we can observe that by introducing the Cysteine to Tyrosine (C/Y) mutation to the WT-AR, receptor activity is increased even in the absence of the co-chaperone FKBP51 (as compared to WT-AR). However, when exposed to FKBP51 (A), the same activity is downregulated (compared to C/Y + Empty Vector). In contrast, in Fig. 14 B, the receptor activity is upregulated by the protein FKBP52.

The Histidine to Tyrosine mutation (H/Y) makes the receptor downregulated in the presence and absence of co-chaperone by approximately 50%, as compared to WT-AR (Fig. 14 C and D).

In E, we can observe that by introducing the Asparagine (691) to Serine (N691S) mutation to the WT-AR, receptor activity is increased by approximately 200% in the absence of FKBP51 (as compared to WT-AR). However, when exposed to the co-chaperone, the same activity is downregulated by approximately 200% (when compared to N691S + Empty Vector). In contrast, in the absence of FKBP52 (F), receptor activity is similar to

that of WT-AR. Moreover, when the co-chaperone 52 is present, a small increment in activity can be seen in the mutant.

G shows that the mutation of Asparagine (692) to Serine (N692S) increases the activity of the receptor by 100% in absence of FKBP51. However, the presence of co-chaperone, negatively regulates the activity of the receptor, making it similar to that of WT-AR. In an alike way, the mutation seems to increase activity of the receptor in the absence of co-chaperone 52, but when this is present, the activity is downregulated back to WT-AR activity (H).

Fig. 14 I and J show the result in reporter expression after introducing the amino acid substitution of a Glutamine for a Valine (Q/V). In I, it can be observed an increase in reporter activity of approximately 50-100% after the mutation, as compared to the WT-AR. However, when FKBP51 is introduced, the activity is decreased almost to the same as WT-AR. Similarly, in J, it can be observed an increment in activity of approximately 100% in the absence of FKBP52. Subsequently, when FKBP52 is added, the reporter activity is decreased to the same amount as WT-AR + FKBP52.

The mutant combination VC/LY makes the receptor downregulated in the presence and absence of co-chaperone by approximately 50%, as compared to WT-AR (Fig. 14 K and L).

The mutant combination HN/YS shows a similar effect as the one seen with the individual mutant N691S (Fig. 14 E and F). In the absence of the co-chaperones FKBP51 (M), an increment in receptor activity can be observed, but when 51 is introduced, the activity goes down to the level of WT-AR. As for figure N, there is increased reporter activity

without FKBP52, but when it is introduced, the activity goes up for about 200% more than WT-AR.

O and P show the results of the mutant combination NQ/SV. Here, we can observe that in the absence of either of the co-chaperones FKBP51 and FKBP52, there is an increment in receptor regulation. However, when the co-chaperones are introduced, opposite effects are shown. 51 (O) negatively regulates the activity of the receptor, lowering the activity to the same levels of WT-AR. While with 52 (P), receptor regulation is up by 200% compared with WT-AR + FKBP52.

Fig. 14 Q and R show that the mutant combination VC/HN makes the receptor downregulated in the presence and absence of co-chaperone, as compared to WT-AR.

S and T, show the result in reporter expression after introducing the mutant combination NQ/VC. In S, it can be observed an increase in reporter activity of approximately 100% after the mutation, as compared to the WT-AR. Contrary, in figure T, it can be observed a decreased in activity of approximately 100%, as compared to WT-AR + FKBP52, while in the absence of co-chaperone, the activity remains close to WT-AR + Empty Vector.

After introducing the mutant combination NQ/HN, it can be observed an increase in reporter activity, as compared to the WT-AR. In figure U, in the absence of FKBP51, the receptor regulation is increased by 300%. However, when 51 is present, the activity is downregulated to the levels of WT-AR. Figure V shows a higher receptor activity than that of WT-AR, with no significant difference between the presence or absence of the co-chaperone FKBP52.

The mutant VCHNNQ/LYYSSV combines all the different individual mutants previously discussed in figures A through V. The combination increases the reporter activity in the absence of either of the co-chaperones 51 or 52, and when exposed to them, the receptor activity is downregulated.

Consequently, to demonstrate the co-expression of AR and the FKBP co-chaperones, a western blot analysis was performed. As shown in figure 15, the protein lysates confirm the presence of the proteins of interest.

In Chapter 2, we demonstrated that changes to the H1-H3 loop on the hAR to make it similar to the loop on the gpGR affected receptor activity. Likewise, after making the hAR H1-H3 loop look like the loop of the hGR, we corroborated that this loop is important for receptor regulation, and that differences within this surface change the ability of the receptor to be regulated by the FKBP cochaperones. Since the activities of AR and GR are similarly modulated by FKBP51 and 52, these data also suggest that this loop is relevant in the regulation of androgen and glucocorticoid signaling.

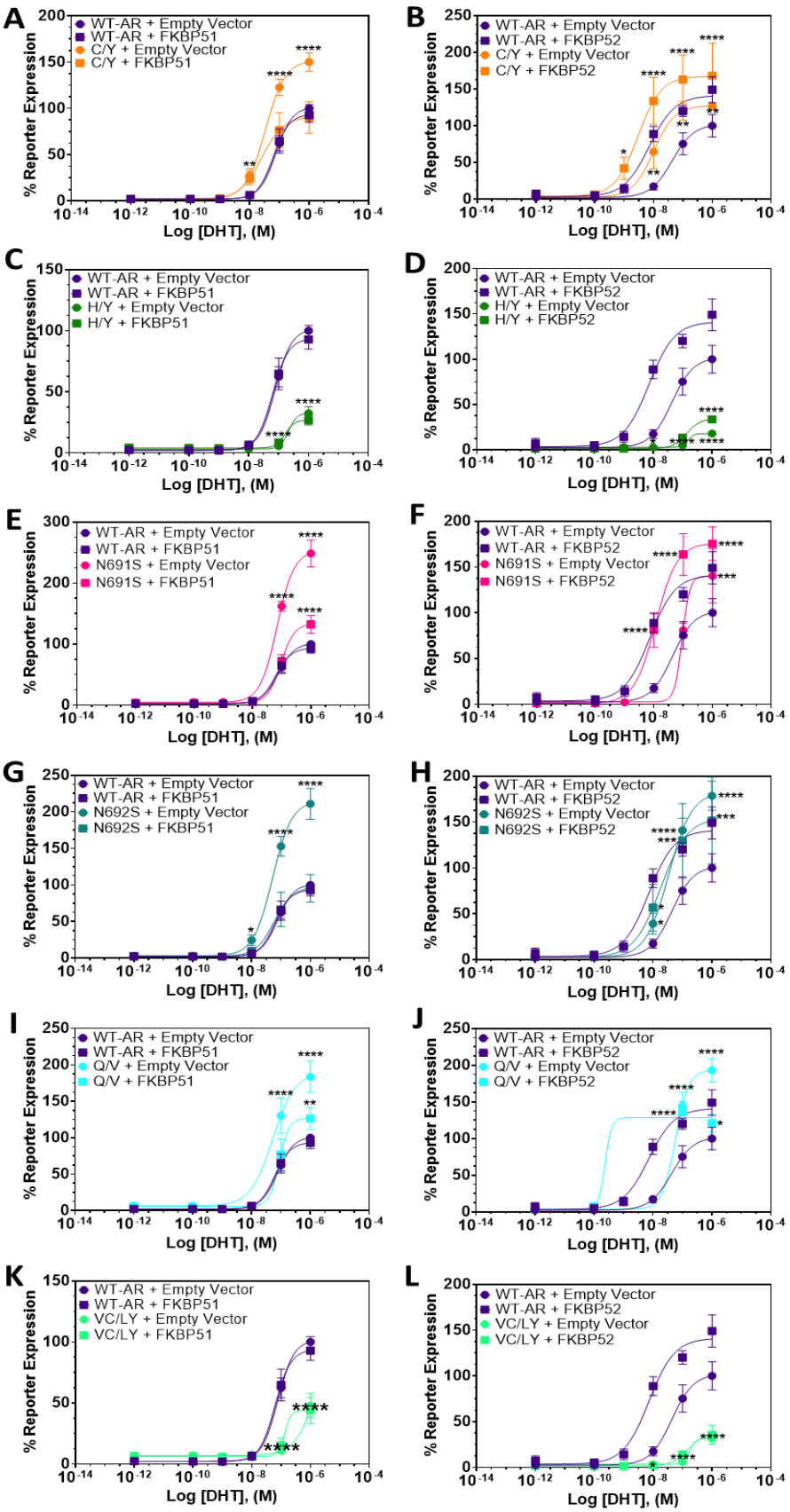

Fig. 14. hAR to hGR Mutants in the absence and presence of FKBP51 or FKBP52

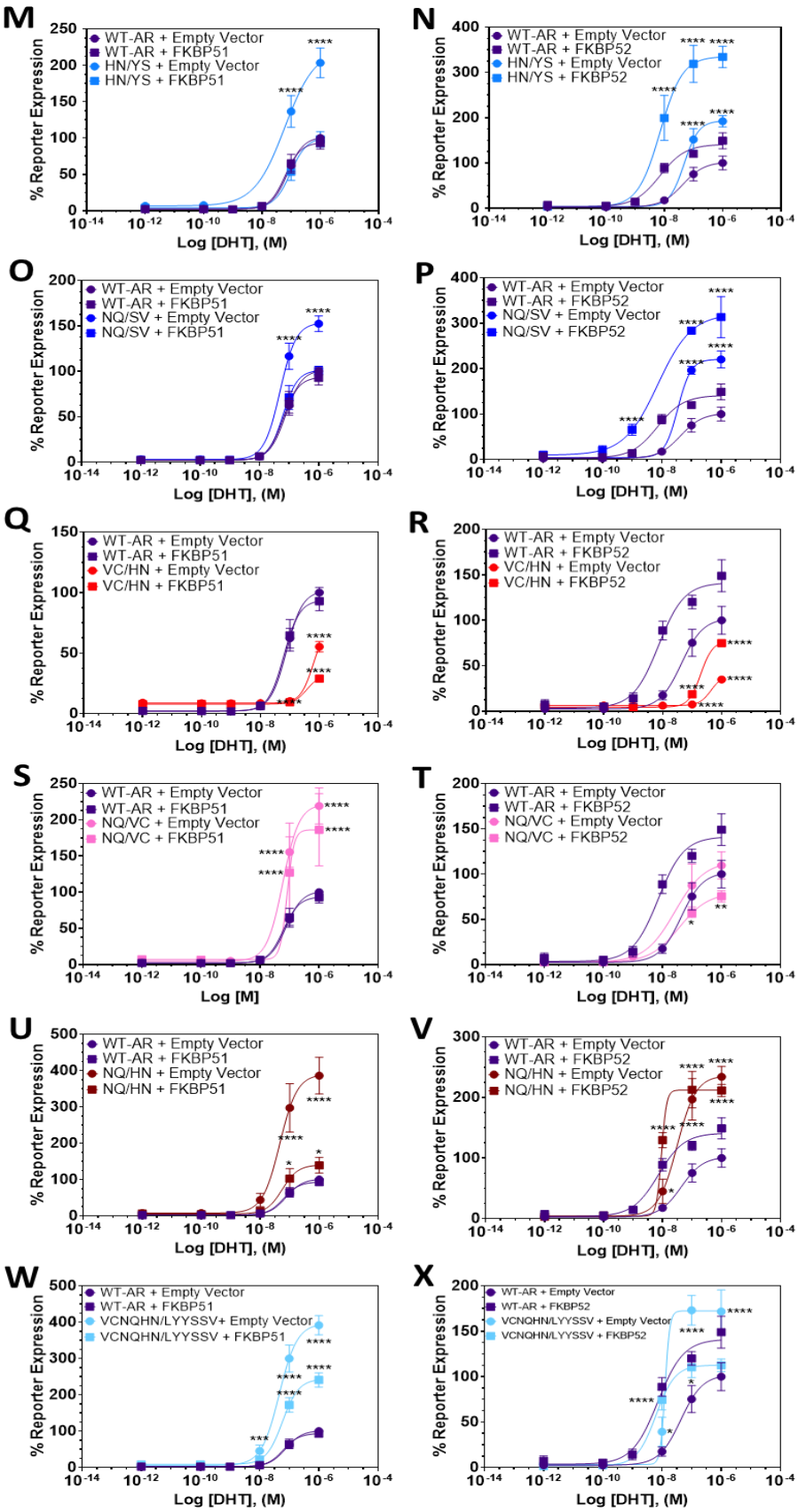

Fig. 14. hAR to hGR Mutants in the absence and presence of FKBP51 or FKBP52

Fig. 14. hAR to hGR Mutants in the absence and presence of FKBP51 or FKBP52

(continuation). *The graphs above represent the mean reporter expression of three independent experiments for WT-AR and mutant hAR in the presence of FKBP52 (FKBP52), FKBP51 (FKBP51) or absence (Empty Vector) of chaperone. Yeast S. cerevisiae were transformed with a lacZ reporter plasmid, an expression plasmid for FKBP51 or FKBP52, and an expression plasmid for wild-type hAR or hAR to hGR mutant and dosed with increasing concentrations of DHT. Statistical analysis was done using a 2-way ANOVA with Dunette's multiple comparisons test.* **********, $P \leq 0.0001$; ********, $P \leq 0.0005$; ******** $P \leq 0.005$; ******* $P \leq 0.05$.

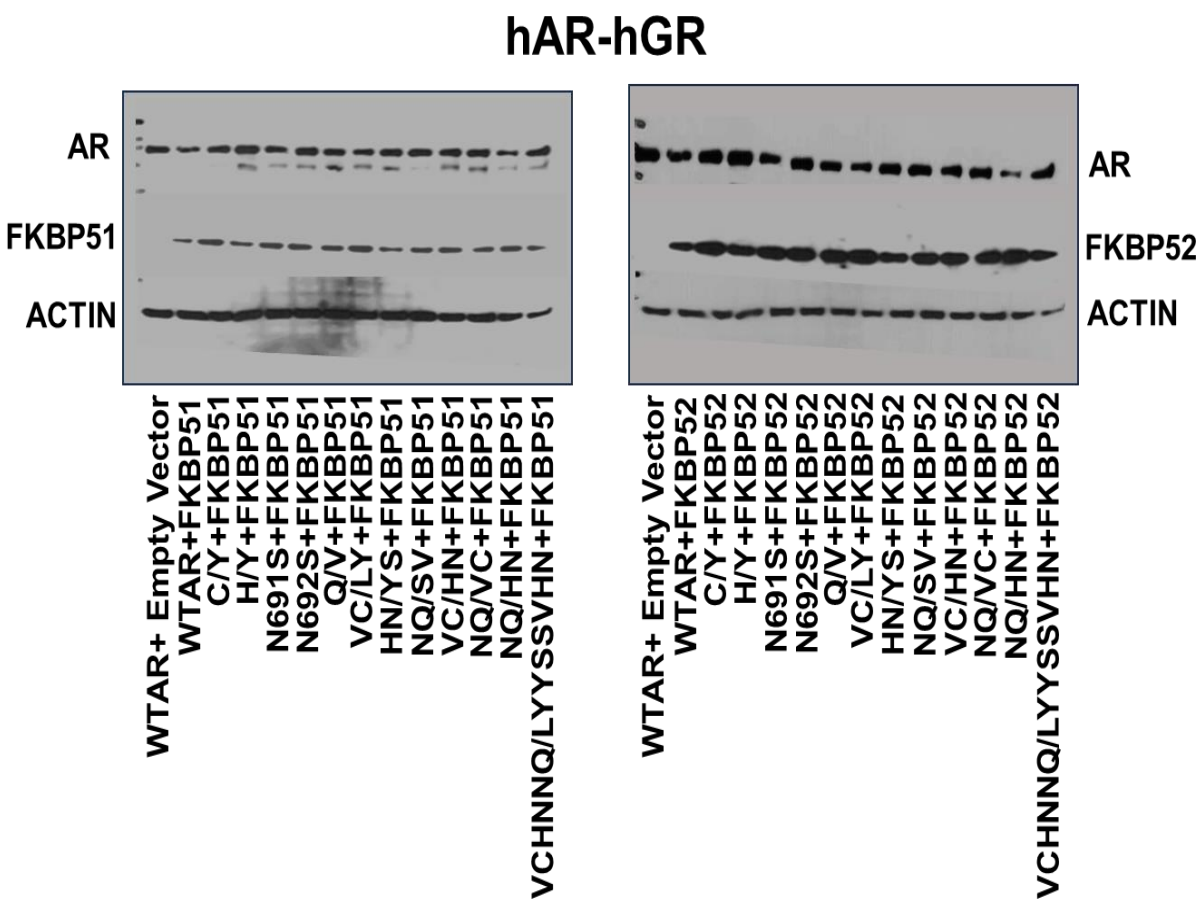

Fig. 15 Validation of the co-expression of proteins of interest. *To validate the co-expression of AR and the FKBP cochaperones 51 and 52in our assays, we performed a Western blot analysis targeting the proteins of interest. Yeast cells were grown in selective media lacking the amino acids Leucine, Uracil and Tryptophan for 18 hrs., and lysed subsequently. Protein lysates were electrophoresed and transfer to a nitrocellulose membrane. Finally, the membrane was incubated with antibodies targeting AR, FKBP52 and FKBP51. As shown, all lysates tested positive for the presence of the proteins of interest.*

CHAPTER 4: CORROBORATE THE YEAST-BASED FINDINGS AND ASSESS THE MUTANTS OF INTEREST FOR FUNCTIONAL EFFECTS ON FKBP REGULATION IN HUMAN CELLULAR MODEL SYSTEMS.

4.1 Rationale

In chapters 2 and 3, we identified critical residues within the ligand binding domain (LBD) of the androgen receptor (AR) helix 1 to 3 (H1-H3) loop capable of affecting receptor regulation. To corroborate these results, the mutants were assessed on a higher model system like human cells.

4.2 Materials and Methods

4.2.1 Mammalian Cell Reporter Assays

The experimental procedure will involve 52 knock-out (KO) or 51KO HeLa cells seeded into a 6-well plate at a density of $2x10^5$ cells/well. The following day, cells will be transfected with plasmids each coding for AR-LBD H1-H3 loop mutants, *lacZ* reporter plasmid, an expression plasmid for FKBP51 or 52, and an expression plasmid. As a control, a set of wells will also be transfected with the wild-type AR plasmid. 24 hours following the transfection, cells will be dosed with increasing concentrations of DHT. After approximately 16 hours of incubation with hormone, cells will be lysed with 100µL mammalian protein extraction reagent (M-PER) supplemented with HALT protease inhibitor. After clarifying the lysates, AR-mediated luciferase expression will be quantified by mixing 40µL of cell lysate with 100µL of luciferase assay reagent (Bright-Glo) in a 96-well plate and incubated at room temperature for 5 minutes. β-galactosidase expression will be quantified by adding 20µL cell lysate with 100µL of Tropix Gal-Screen in a 96-well plate at room temperature for 2 hours. Quantification of luminescence will be conducted in a microplate luminometer with luminescence measured as Relative Light Units (RLU) and normalized to β-galactosidase activity to control transfection efficiency.

4.2.2 Western Analysis

WT, 52KO and 51 KO HeLa cells previously transfected with plasmids coding for AR-LBD H1-H3 loop mutants, were plated at $3x10^5$ cells per well, in a 6-well culture plate in 1.5mL of appropriate growth media and allowed to incubate at 37ºC with 5% CO2. The following day the cells were treated with 10nM DHT or EtOH and allowed to incubate for 16-18 hours at 37ºC with 5% CO2. After 16-18 hours, the cells were lysed and collected. A Coomassie Plus (Bradford) Protein Assay (ThermoScientific) was conducted to determine the amount of protein concentration in each sample. The proteins (lysates) were then loaded onto Invitrogen Bolt™ 4-12% Bis-Tris Plus Precast gels (ThermoFisher Scientific). Once the proteins were separated by electrophoresis, the gel was transferred to Immobilon® -P Polyvinylidene Difluoride Membrane (PVDF, Millipore Sigma). The membranes were blocked with 5% nonfat dry milk in 1x Tris-Buffered Saline (blocking buffer) for 30 minutes on a shaking plate at room temperature.

The primary antibodies that were used are the following: mouse monoclonal anti-FKBP52 Hi52D (1:5000), mouse monoclonal anti-GAPDH (1:3000; loading control; Santa Cruz Biotechnology), and rabbit polyclonal anti-AR (1:1000; Santa Cruz Biotechnology). Primary antibodies were diluted in blocking buffer and allowed to incubate with appropriate membranes for 1 hour on a plate shaker at room temperature. The membranes were then washed 5 times with TBS-Tween 20 for 5 minutes on a plate shaker at room temperature. Secondary antibodies consisting of alkaline-phosphatase conjugated goat anti-mouse IgG (1:5000; Southern Biotech), and alkaline-phosphatase conjugated goat anti-rabbit IgG (1:5000; Southern Biotech), were diluted in blocking buffer and allowed to incubate with appropriate membranes for 1 hour on a plate shaker at room

temperature. The membranes were washed 5 times with TBS-Tween 20 for 5 minutes on a plate shaker at room temperature. The membranes were finally developed with Immun-Star™ AP substrate (BioRad) and exposed on CL-X Posure™ film (Thermo Scientific) for detection of antibodies.

4.2.3 Statistical Analysis

The descriptive statistics were calculated, and the graphs plotted using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad. Statistical significance was determined by Two-way ANOVA followed by Dunnett's multiple comparisons test.

4.3 Results

Our current data obtained from yeast-based assays shows that the H1-H3 loop is likely a relevant FKBP regulatory surface for the steroid hormone receptors AR and GR. Therefore, we randomly selected one mutant per class (hAR-gpGR and hAR-hGR) to corroborate these results on WT, 52KO and 51KO HeLa cells. The selected mutants for this assay were NQ/SV, as a representant for the hAR-hGR class of mutants, and VCANQ/IHSTS for the hAR-gpGR mutants' class. HeLa cells (WT or KO) were transfected with a plasmid coding for WT-AR or mutants and dosed with increasing concentrations of DHT to generate full hormone dose-response curves (Fig 16). Simultaneously, protein lysates were analyzed by western blot to verify transfection efficiency (Fig. 17).

Figure 16, shows the activity of the mutants NQ/SV and VCANQ/IHSTS compared with that of WT-AR. A, shows the effects of the mutants NQ/SV and VCANQ/IHSTS in the presence of FKBP52 or FKBP51, as obtained in the yeast-based assays. On B, the

functional effects of both mutants were studied on WT-HeLa cells. As shown, the results obtained in the mammalian assays are similar to those from the yeast-based assays (Fig. 11 M, N). The mutant combination VCANQ/IHSTS remains the same as that of WT-AR in the presence of the FKBP co-chaperones. Whereas the mutant combination NQ/SV is upregulated. However, on the yeast assays, the mutant was upregulated by the presence of FKBP52, while downregulated by FKBP51. It is well known that both co-chaperones compete for binding of the AR, which can be seen in the experiments with the WT-HeLa cells.

Experiments run on 52KO-HeLa cells (Fig. 16 C) corroborated the effects of the mutants as seen on yeast. Here we can observe that the activity of both mutants is almost identical to that of WT-AR. On yeast-based assays, the mutants were upregulated in the absence of any of the co-chaperones but downregulated back to the activity of WT-AR in the presence of FKBP51.

51KO-HeLa cells (Fig. 16 D) show that in the presence of FKBP52 but not 51, the mutant NQ/SV is upregulated in yeast and mammalian assays. Whereas the activity of the mutant VCANQ/IHSTS was downregulated back to that of WT-AR. These corroborates the results shown in the yeast-based assays.

Next, a western blot with the lysates obtained from the dose-response assays on the HeLa cells was conducted. Fig. 17 shows the results obtained from this assay, where we can observe that in WT-HeLa (A), both co-chaperones are expressed, however AR is only present when co-expressed after transfection. It can also be seen that the 52KO line (B) does not express FKBP52, but it continues to express 51. On the other hand, the 51KO cell line (C), does not express 51 but it expresses 52. We can also observe a difference

in the protein expression levels, where the expression of the NQ/SV mutant is higher than that of WT-AR or VCANQ/IHSTS for all the different HeLa cell lines. This suggests that NQ/SV makes AR expression more stable.

These results provide further confirmation that the H1-H3 loop is important for receptor regulation.

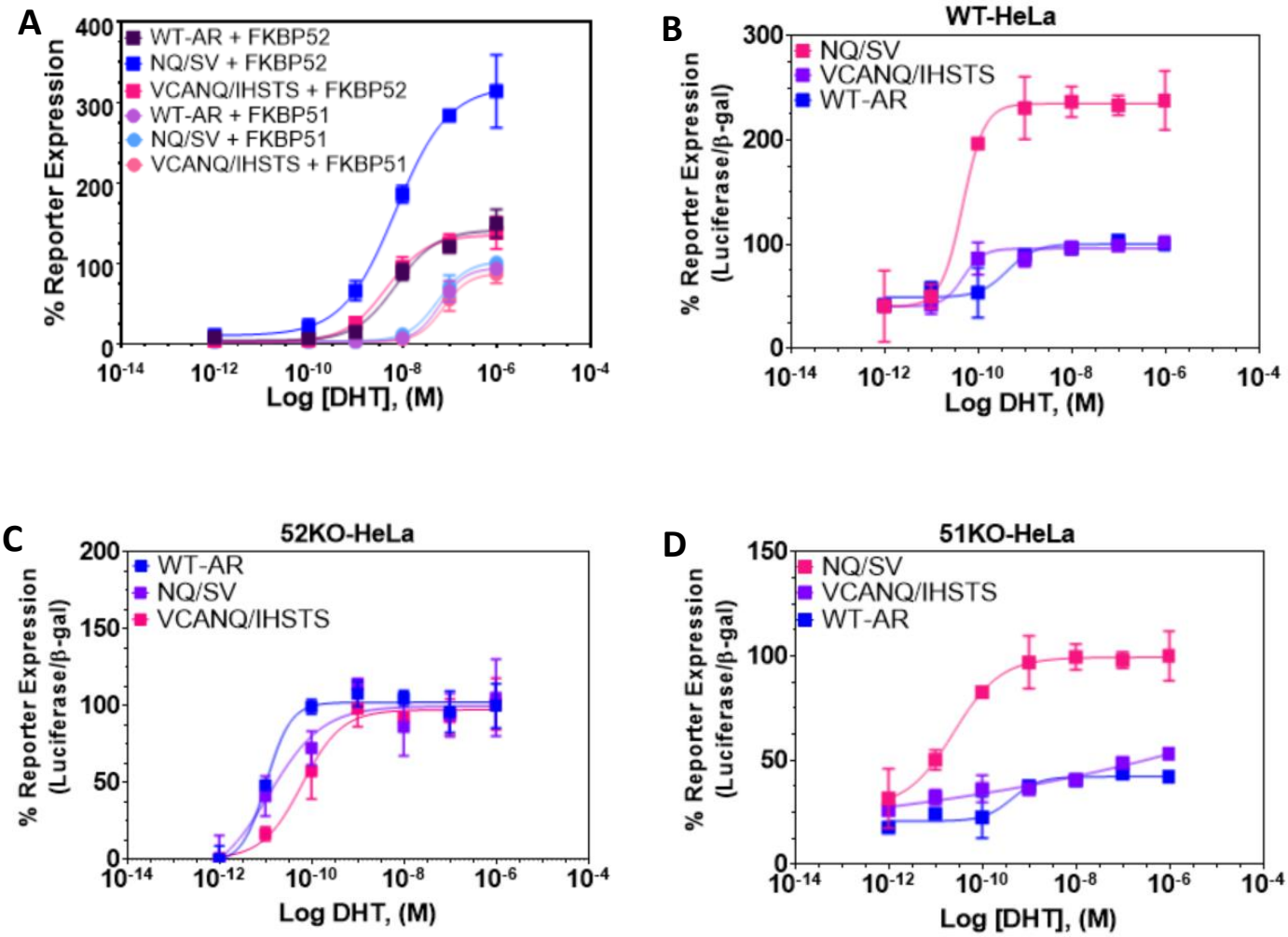

Fig. 16. Mutations within the H1-H3 loop effectively interfere with receptor regulation.

Fig. 16 continuation. *The graphs show the results of two independent experiments where WT, 51KO, and 52KO HeLa cells were seeded on a 6-well plate at a density of* $3x10^5$ *cells per well, and transfected with a β-galactosidase expression plasmid (pDS-371, 50 ng per well), a mammalian expression MMTV-Luc-Reporter plasmid (pDS-372, 800 ng per well), a pCI-Neo mammalian expression vector expressing AR or the hAR-gpGR mutant VCANQ/IHSTS or hAR-hGR mutant NQ/SV (pDS-923/mutant, 800 ng per well), and an empty pCI-Neo mammalian expression vector (pDS-404, 800 ng per well). After 24 hrs, cells were treated with ETOH (0.01%) and increasing doses of DHT and incubated for 16-20 hrs. Lysates were collected and used to measure luciferase expression. The reporter expression was normalized against transfection efficiency by dividing luciferase relative light units by β-galactosidase relative light units. Statistical analysis was done using a One-way ANOVA with Dunette's multiple comparisons test (P<0.05). Afterwards, lysates were analysed via western blotting to corroborate the expression of AR, FKBP52 or FKBP51.*

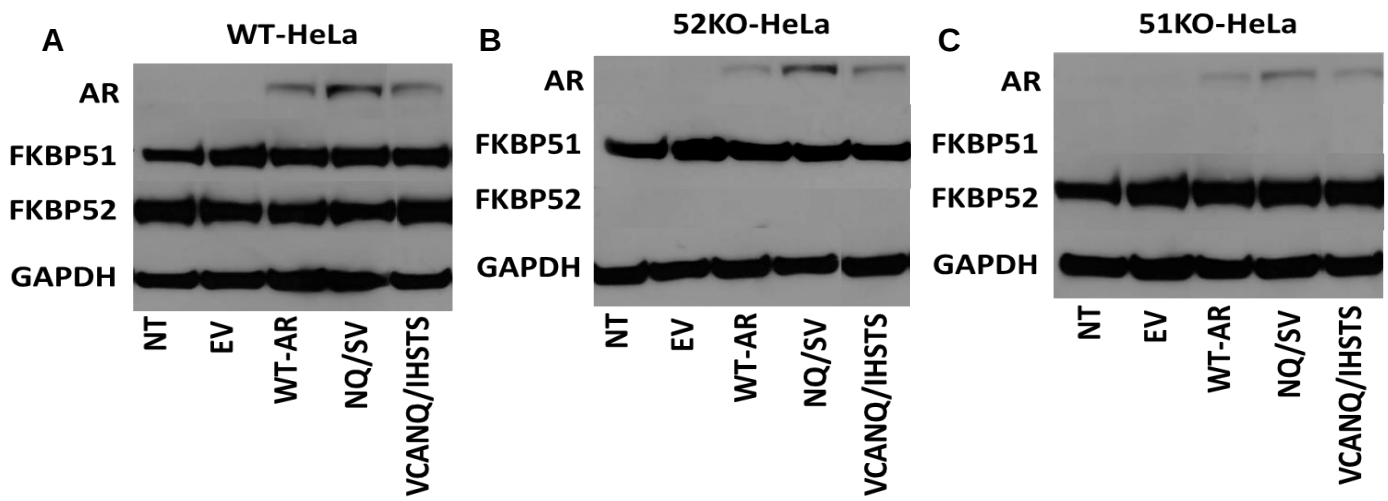

Fig. 17. Validation of the co-expression of proteins of interest. *To validate the co-expression of AR and the FKBP cochaperones 51 and 52 in our assays in HeLa cells (WT, 52KO and 51KO), we performed a Western blot analysis targeting the proteins of interest. Protein lysates were electrophoresed and transfer to a nitrocellulose membrane. Finally, the membrane was incubated with antibodies targeting AR, FKBP52 and FKBP51. As shown, all lysates tested positive for the presence of the proteins of interest.*

CHAPTER 5: CONCLUSIONS

Prostate Cancer initiation and progression are dependent upon androgen receptor regulated genes. Functional receptor conformation requires the cooperation of chaperone and cochaperone proteins including the immunophilins FKBP52 and FKBP51. FKBP52 is known for being a positive regulator of AR, and GR activity, whereas FKBP51 is characterized for differentially regulating the steroid hormone receptor family. Therefore, these two proteins have become potential therapeutic targets for prostate cancer treatment. Androgen Receptor and Glucocorticoid receptor share approximately 50% similarity in the ligand binding domain. However, structural or sequence differences may confer the differences in how FKBP cochaperones regulate their activity. Previous Studies made on guinea pigs, found that 5 amino acid substitutions within the H1-H3 loop of the ligand binding domain that differ from the human glucocorticoid receptor, conferred decreased cortisol-binding affinity. Since the androgen receptor also differs from the glucocorticoid receptor by those same amino acids, we hypothesized that this loop might be responsible for receptor regulation among glucocorticoid and androgen receptor.

Previous studies have identified the BF3 surface as an AR-specific regulatory site for FKBP52, and it is likely that there is another common FKBP regulatory surface among all the regulated receptors, like the H1-H3 loop. Furthermore, GR is known to be more hypersensitive to FKBP52 regulation, and recent research has shown that mutations within the GR H1-H3 loop affect FKBP-mediated receptor activities.

In this project, we successfully conducted site-directed mutagenesis to identify the residues within the hAR H1-H3 loop that are critical for FKBP co-chaperone regulation. Taking advantage of the distinct GR hypersensitivity to FKBP52, two classes of functional mutants were generated to make the human AR H1-H3 loop more like guinea pig GR or

human GR. In addition, yeast-based reporter assays were performed to assess the role and relevance of those mutations in receptor activity. Finally, these results were corroborated by randomly choosing one mutant of interest to represent each of the classes with those obtained from a higher model system like the HeLa cells.

To determine whether the hAR mutants affected FKBP-mediated receptor function, reporter gene expression was measured by dosing yeast cultures with increasing concentrations of DHT to generate full hormone dose-response curves for the WT-AR strain, mutant strains, and vector control strain. Subsequently, a western blot was performed to validate the co-expression of AR, and the FKBP co-chaperones 51 and 52 on protein lysates obtained from the yeast assays.

In Chapter 2, we demonstrated that changes to the H1-H3 loop on the hAR to make it similar to the loop on the gpGR affected receptor activity. The mutant C/H was upregulated even in the absence of the FKBP co-chaperones, conversely, the mutant FKBP52 hyperregulated the receptor activity by approximately 200X more than the WT-AR. The mutant Q/S showed no significant difference between the presence or absence of the co-chaperone FKBP51. In contrast, when FKBP52 was present, the activity of the receptor was downregulated. VCA/HIS lost receptor activity probably due to protein misfolding. The mutant combination VCANQ/IHSTS was upregulated even in the absence of co-chaperone. However, when the FKBP co-chaperones were introduced, receptor activity was negatively regulated. This effect was shown with the rest of the individual mutants.

Likewise, after making the hAR H1-H3 loop look like the loop of the hGR in chapter 3, we corroborated that this loop is important for receptor regulation, and that differences within

this surface change the ability of the receptor to be regulated by the FKBP cochaperones. The mutants H/Y, VC/LY, and VC/HN are downregulated with and without co-chaperones. N692S, Q/V, NQ/VC, NQ/HN, and VCHNNQ/LYYSSV were upregulated in absence of either of the co-chaperones but downregulated in their presence. The mutant NQ/SV was upregulated with and without either of the co-chaperones. Moreover, when FKBP52 was co-expressed, receptor activity increased 200X more. In contrast, FKBP51 presence negatively regulated receptor activity. The rest of the mutants behave similarly to the mutant combination NQ/SV. Since the activities of AR and GR are similarly modulated by FKBP51 and 52, these data also suggest that this loop is relevant in the regulation of androgen and glucocorticoid signaling.

Based on these findings, mammalian reporter assays were conducted to corroborate our findings in a higher vertebrate model system. We randomly selected one mutant per class (NQ/SV to represent the hAR-hGR class, and VCANQ/IHSTS to represent the hAR-gpGR class) to assess their effects on receptor activity on WT, 52KO and 51KO HeLa cells. We verified that amino acid substitutions within the H1-H3 loop effectively interfere with receptor regulation. Experiments conducted on WT-HeLa cells showed that the activity of the mutant NQ/SV is hyperregulated as compared to that of WT-AR. Whereas the activity of VCANQ/IHSTS remained similar to WT-AR. This is consistent with the results seen in the yeast-based assays, where it was shown that in the presence of FKBP52, the mutant NQ/SV was hyperregulated, while VCANQ/IHSTS receptor activity was negatively regulated and restored back to the same activity as WT-AR. Correspondingly, assays conducted on 51 and 52 KO cells demonstrated that in the absence of the co-chaperones, the activity of the mutant combination VCANQ/IHSTS remain similar to that of WT-AR.

As for NQ/SV, in the absence of 52, it was downregulated, while in the absence of 51, it was still upregulated. The hyperregulation in the WT-HeLa cells can be explained by the fact that 52 and 51 compete for binding to the AR (67). As for the mutant VCANQ/IHSTS, on yeast assays, we saw an increased activity in the absence of co-chaperones, but when 52 or 51 were introduced, the activity of the mutated receptor went back to the same levels as WT-AR, similar to what is shown in the WT-HeLa cells.

Our current data shows that, mechanistically, the H1-H3 loop is a relevant regulatory surface for the steroid hormone receptors AR and GR and that differences within this surface may change the ability of the receptor to be regulated by the FKBP cochaperones. Taken together, these results suggest that the H1- H3 loop may represent a novel target surface for the simultaneous inhibition of AR and GR.

These results coincide with those obtained by Cluning et al. The team observed that by altering the H1-H3 loop of the GR, FKBP-regulated activity was affected, which lead them to conclude that the GR H1-H3 loop acts as a regulatory surface for the FKBP co-chaperones by creating conformational changes that impact receptor activity.

Mutants within the H1-H3 loop have been implicated in androgen insensitivity syndrome (AIS), either partial (PAIS) or complete (CAIS) (Fig. 18) (68). Examples of these are the mutant substitutions V685I, and H689P which have been found to be present in patients with complete androgen insensitivity. Whereas the mutants C686R, and A687V have been found in patients with partial androgen insensitivity. Interestingly, the mutant V685I was one of the amino acid substitutions studied in this project, which showed an upregulated receptor activity in the absence of either of the co-chaperones FKBP51 or 52 but was downregulated by the presence of the FKBP co-chaperones.

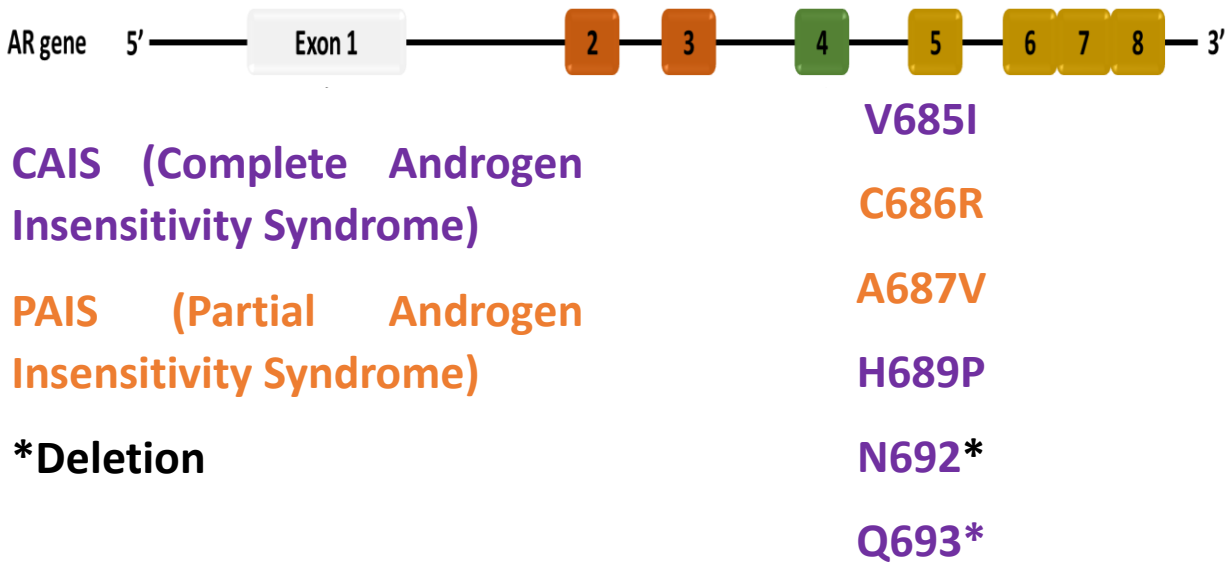

Fig. 18. Androgen Receptor H1-H3 loop gene mutations. *By consulting the Androgen Receptor Gene Mutations database, we selected the amino acid substitutions within the H1-H3 loop that are associated with androgen insensitivity syndrome.*

Furthermore, mutations within the BF3 surface have also been implicated in AIS (69). At the same time, mutations to the BF3 surface can also interfere with the AF2 surface, affecting co-activator interaction (27). The BF3 surface has been studied by our lab as an AR-specific regulatory site for FKBP52. In this project, we demonstrated that there is another site on the AR LBD that is important for FKBP receptor regulation. However, it Is still unclear if this site serves as a direct interaction surface for the co-chaperones. It is likely that by changing the AR H1-H3 loop, we could be affecting chaperone binding by way of the BF3 or AF2 surfaces allosterically.

To further investigate the effects of the H1-H3 loop mutants, it would be necessary to express the FKBP51 and FKBP52 back in the KO cell lines to contrast the receptor activity in the presence and absence of the cochaperones in human cells. It will be interesting to

elucidate if by co-expressing the FKBP co-chaperones in the KO cell lines, the activity of the receptor remains comparable to the results observed on yeast-based assays.

Computational analysis could be used to predict the way that these mutants may affect protein conformation. Due to the closeness of the mutants with the BF3 surface, it is possible that they create changes in the spatial orientation of critical residues for the chaperone complex.

Another path to study will be to assess relevant mutants for altered response to previously identified FKBP52 targeting drugs, like MJC13 and GMC1. These drugs are believed to interfere with the chaperone complex binding to AR by way of the BF3 surface (28, 70). This analysis could also help elucidate if the H1-H3 loop mutations affect the spatial relations within the chaperone complex.

Finally, to confirm if this loop is a putative regulatory surface for all three receptors (AR, GR and PR), it will be necessary to test these mutations on PR models and assess for functional effects.

REFERENCES

1. Chen F-Z, Zhao X-K. 2013. Prostate cancer: current treatment and prevention strategies. Iran Red Crescent Med J2013/04/05. 15:279–284.
2. Litwin MS, Tan H-J. 2017. The Diagnosis and Treatment of Prostate Cancer: A Review. JAMA 317:2532–2542.
3. American Cancer Society. 2016. Prostate Cancer What is prostate cancer ? Am Cancer Soc 88.
4. Shen MM, Abate-Shen C. 2010. Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev 24:1967–2000.
5. Snaterse G, Visser JA, Arlt W, Hofland J. 2017. Circulating steroid hormone variations throughout different stages of prostate cancer. Endocr Relat Cancer 24:R403–R420.
6. Mostaghel EA. 2013. Steroid hormone synthetic pathways in prostate cancer. Transl Androl Urol Vol 2, No 3 (September 12, 2013) Transl Androl Urol (Prostate Cancer).
7. Ganguly S, Naik D, Muskara A, Mian OY. 2021. The Nexus of Endocrine Signaling and Cancer: How Steroid Hormones Influence Genomic Stability. Endocrinology 162:bqaa177.
8. Saha S, Dey S, Nath S. 2021. Steroid Hormone Receptors: Links With Cell Cycle Machinery and Breast Cancer Progression . Front Oncol .
9. Tan ME, Li J, Xu HE, Melcher K, Yong EL. 2015. Androgen receptor: Structure, role in prostate cancer and drug discovery. Acta Pharmacol Sin 36:3–23.
10. Xu J, Qiu Y. 2016. Role of androgen receptor splice variants in prostate cancer

metastasis. Asian J Urol 3:177–184.

11. Wilson EM. 2010. Androgen receptor molecular biology and potential targets in prostate cancer. Ther Adv Urol 2:105–117.
12. Heinlein CA, Chang C. 2004. Androgen receptor in prostate cancer. Endocr Rev 25:276–308.
13. Morova T, McNeill DR, Lallous N, Gönen M, Dalal K, Wilson DM, Gürsoy A, Keskin Ö, Lack NA. 2020. Androgen receptor-binding sites are highly mutated in prostate cancer. Nat Commun 11:832.
14. Lonergan P, Tindall D. 2011. Androgen receptor signaling in prostate cancer development and progression. J Carcinog 10:1–28.
15. Sekhoacha M, Riet K, Motloung P, Gumenku L, Adegoke A, Mashele S. 2022. Prostate Cancer Review: Genetics, Diagnosis, Treatment Options, and Alternative Approaches. Molecules 27.
16. Feng Q, He B. 2019. Androgen Receptor Signaling in the Development of Castration-Resistant Prostate Cancer . Front Oncol .
17. Formaggio N, Rubin MA, Theurillat J-P. 2021. Loss and revival of androgen receptor signaling in advanced prostate cancer. Oncogene 40:1205–1216.
18. C. Guy N, A. Garcia Y, B. Cox M. 2015. Therapeutic Targeting of the FKBP52 Co-Chaperone in Steroid Hormone Receptor-Regulated Physiology and Disease. Curr Mol Pharmacol 9:109–125.
19. Tavassoli P. 2019. Androgen Receptor Modulation by non-Androgenic Factors and the Basal Transcription Factor TAF1.
20. Zhou Y, Bolton EC, Jones JO. 2015. Androgens and androgen receptor signaling

in prostate tumorigenesis. J Mol Endocrinol 54:R15–R29.

21. Shafi AA, Cox MB, Weigel NL. 2013. Androgen receptor splice variants are resistant to inhibitors of Hsp90 and FKBP52, which alter androgen receptor activity and expression. Steroids 78:548–554.

22. Fujita K, Nonomura N. 2018. Role of Androgen Receptor in Prostate Cancer: A Review. World J Mens Health.

23. Messner EA, Steele TM, Tsamouri MM, Hejazi N, Gao AC, Mudryj M, Ghosh PM. 2020. The Androgen Receptor in Prostate Cancer: Effect of Structure, Ligands and Spliced Variants on Therapy. Biomedicines.

24. Westaby D, Fenor de La Maza M de LD, Paschalis A, Jimenez-Vacas JM, Welti J, de Bono J, Sharp A. 2022. A New Old Target: Androgen Receptor Signaling and Advanced Prostate Cancer. Annu Rev Pharmacol Toxicol 62:131–153.

25. Fujita K, Nonomura N. 2018. Role of Androgen Receptor in Prostate Cancer: A Review. World J Mens Health 36:288–295.

26. Jehle K, Cato L, Neeb A, Muhle-Goll C, Jung N, Smith EW, Buzon V, Carbó LR, Estebánez-Perpiña E, Schmitz K, Fruk L, Luy B, Chen Y, Cox MB, Braše S, Brown M, Cato ACB. 2014. Coregulator control of androgen receptor action by a novel nuclear receptor-binding motif. J Biol Chem 289:8839–8851.

27. Estébanez-Perpiñá E, Arnold LA, Nguyen P, Rodrigues ED, Mar E, Bateman R, Pallai P, Shokat KM, Baxter JD, Guy RK, Webb P, Fletterick RJ. 2007. A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci U S A 104:16074–16079.

28. De Leon JT, Iwai A, Feau C, Garcia Y, Balsiger HA, Storer CL, Suro RM, Garza

KM, Lee S, Kim YS, Chen Y, Ning YM, Riggs DL, Fletterick RJ, Guy RK, Trepel JB, Neckers LM, Cox MB. 2011. Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proc Natl Acad Sci U S A 108:11878–11883.

29. Patricia Hernández M, Chadli A, Toft DO. 2002. HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. J Biol Chem 277:11873–11881.

30. Connell P, Ballinger CA, Jiang J, Wu Y, Thompson LJ, Höhfeld J, Patterson C. 2001. The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol 3:93–96.

31. Young JC, Agashe VR, Siegers K, Hartl FU. 2004. Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol 5:781–791.

32. Picard D. 2006. Chaperoning steroid hormone action 17.

33. Trepel J, Mollapour M, Giaccone G, Neckers L. 2010. Targeting the dynamic HSP90 complex in cancer 10.

34. Cox MB, Johnson JL. 2011. The Role of p23, Hop, Immunophilins, and Other Co-chaperones in Regulating Hsp90 Function, p. 45–66. *In* Calderwood, SK, Prince, TL (eds.), Molecular Chaperones: Methods and Protocols. Humana Press, Totowa, NJ.

35. Erlejman AG, Lagadari M, Harris DC, Cox MB, Galigniana MD. 2014. Molecular chaperone activity and biological regulatory actions of the TPR-domain immunophilins FKBP51 and FKBP52. Curr Protein Pept Sci 15:205–215.

36. Tao YJ, Zheng W. 2011. Chaperones and the maturation of steroid hormone

receptor complexes. Oncotarget 2:104–106.

37. Wandinger SK, Richter K, Buchner J. 2008. The Hsp90 chaperone machinery. J Biol Chem 283:18473–18477.

38. Ni L, Yang C-S, Gioeli D, Frierson H, Toft DO, Paschal BM. 2010. FKBP51 Promotes Assembly of the Hsp90 Chaperone Complex and Regulates Androgen Receptor Signaling in Prostate Cancer Cells. Mol Cell Biol 30:1243–1253.

39. Murphy PJM, Morishima Y, Chen H, Galigniana MD, Mansfield JF, Simons SS, Pratt WB. 2003. Visualization and mechanism of assembly of a glucocorticoid receptor·Hsp70 complex that is primed for subsequent Hsp90-dependent opening of the steroid binding cleft. J Biol Chem 278:34764–34773.

40. Echeverria PC, Picard Didier D. 2010. Molecular chaperones, essential partners of steroid hormone receptors for activity and mobility. Biochim Biophys Acta - Mol Cell Res 1803:641–649.

41. Prescott J, Coetzee GA. 2006. Molecular chaperones throughout the life cycle of the androgen receptor. Cancer Lett 231:12–19.

42. Storer CL, Dickey CA, Galigniana MD, Rein T, Cox MB. 2011. FKBP51 and FKBP52 in signaling and disease. Trends Endocrinol Metab 22:481–490.

43. Storer Samaniego C, Suh JH, Chattopadhyay A, Olivares K, Guy N, Sivils JC, Dey P, Yumoto F, Fletterick RJ, Strom AM, Gustafsson J-Å, Webb P, Cox MB. 2015. The FKBP52 Cochaperone Acts in Synergy with β-Catenin to Potentiate Androgen Receptor Signaling. PLoS One 10:e0134015.

44. Maeda K, Habara M, Kawaguchi M, Matsumoto H, Hanaki S, Masaki T, Sato Y, Matsuyama H, Kunieda K, Nakagawa H, Shimada M. 2022. FKBP51 and FKBP52

regulate androgen receptor dimerization and proliferation in prostate cancer cells. Mol Oncol 16:940–956.

45. Soto OB, Ramirez CS, Koyani R, Rodriguez-Palomares IA, Dirmeyer JR, Grajeda B, Roy S, Cox MB. 2023. Structure and function of the TPR-domain immunophilins FKBP51 and FKBP52 in normal physiology and disease. J Cell Biochem n/a.

46. Sivils JC, Storer CL, Galigniana MD, Cox MB. 2011. Regulation of steroid hormone receptor function by the 52-kDa FK506-binding protein (FKBP52). Curr Opin Pharmacol 11:314–319.

47. Galat A. 2003. Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity--targets--functions. Curr Top Med Chem 3:1315–1347.

48. Blatch GL, Lässle M. 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays 21:932–939.

49. Radanyi C, Chambraud B, Baulieu EE. 1994. The ability of the immunophilin FKBP59-HBI to interact with the 90-kDa heat shock protein is encoded by its tetratricopeptide repeat domain. Proc Natl Acad Sci U S A 91:11197–11201.

50. Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I. 2000. Structure of TPR Domain–Peptide Complexes: Critical Elements in the Assembly of the Hsp70–Hsp90 Multichaperone Machine. Cell 101:199–210.

51. Russell LC, Whitt SR, Chen MS, Chinkers M. 1999. Identification of conserved residues required for the binding of a tetratricopeptide repeat domain to heat shock protein 90. J Biol Chem 274:20060–20063.

52. Lebeau MC, Massol N, Herrick J, Faber LE, Renoir JM, Radanyi C, Baulieu EE. 1992. P59, an hsp 90-binding protein. Cloning and sequencing of its cDNA and preparation of a peptide-directed polyclonal antibody. J Biol Chem 267:4281–4284.

53. Chambraud B, Rouvière-Fourmy N, Radanyi C, Hsiao K, Peattie DA, Livingston DJ, Baulieu EE. 1993. Overexpression of p59-HBI (FKBP59), full length and domains, and characterization of PPIase activity. Biochem Biophys Res Commun 196:160–166.

54. Riggs DL, Roberts PJ, Chirillo SC, Cheung-flynn J, Prapapanich V, Ratajczak T, Gaber R, Picard D, Smith DF. 2003. The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo 22.

55. Tranguch S, Cheung-Flynn J, Daikoku T, Prapapanich V, Cox MB, Xie H, Wang H, Das SK, Smith DF, Dey SK. 2005. Cochaperone immunophilin FKBP52 is critical to uterine receptivity for embryo implantation. Proc Natl Acad Sci U S A 102:14326–14331.

56. Cheung-Flynn J, Prapapanich V, Cox MB, Riggs DL, Suarez-Quian C, Smith DF. 2005. Physiological role for the cochaperone FKBP52 in androgen receptor signaling. Mol Endocrinol 19:1654–1666.

57. Riggs DL, Cox MB, Tardif HL, Hessling M, Buchner J, Smith DF. 2007. Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. Mol Cell Biol2007/10/15. 27:8658–8669.

58. Bracher A, Kozany C, Hähle A, Wild P, Zacharias M, Hausch F. 2013. Crystal structures of the free and ligand-bound FK1-FK2 domain segment of FKBP52

reveal a flexible inter-domain hinge. J Mol Biol 425:4134—4144.

59. Cox MB, Riggs DL, Hessling M, Schumacher F, Buchner J, Smith DF. 2007. FK506-Binding Protein 52 Phosphorylation: A Potential Mechanism for Regulating Steroid Hormone Receptor Activity. Mol Endocrinol 21:2956–2967.

60. Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE. 2002. Crystal Structure of the Glucocorticoid Receptor Ligand Binding Domain Reveals a Novel Mode of Receptor Dimerization and Coactivator Recognition. Cell 110:93–105.

61. Keightley M-C, Curtis AJ, Chu S, Fuller PJ. 1998. Structural Determinants of Cortisol Resistance in the Guinea Pig Glucocorticoid Receptor*. Endocrinology 139:2479–2485.

62. Fuller PJ, Smith BJ, Rogerson FM. 2004. Cortisol resistance in the New World revisited. Trends Endocrinol Metab 15:296–299.

63. Keightley M-C, Fuller PJ. 1994. Unique sequences in the guinea pig glucocorticoid receptor induce constitutive transactivation and decrease steroid sensitivity. Mol Endocrinol 8:431–439.

64. Keightley M-C, Fuller PJ. 1995. Cortisol resistance and the guinea pig glucocorticoid receptor. Steroids 60:87–92.

65. Cluning C, Ward BK, Rea SL, Arulpragasam A, Fuller PJ, Ratajczak T. 2013. The helix 1-3 loop in the glucocorticoid receptor LBD is a regulatory element for FKBP cochaperones. Mol Endocrinol2013/05/17. 27:1020–1035.

66. Balsiger HA, Cox MB. 2009. Yeast-Based Reporter Assays for the Functional

Characterization of Cochaperone Interactions with Steroid Hormone Receptors, p. 141–156. *In* McEwan, IJ (ed.), The Nuclear Receptor Superfamily: Methods and Protocols. Humana Press, Totowa, NJ.

67. Yong W, Yang Z, Periyasamy S, Chen H, Yucel S, Li W, Lin LY, Wolf IM, Cohn MJ, Baskin LS, Sánchez ER, Shou W. 2007. Essential Role for Co-chaperone Fkbp52 but Not Fkbp51 in Androgen Receptor-mediated Signaling and Physiology. J Biol Chem 282:5026–5036.
68. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. 2012. The androgen receptor gene mutations database: 2012 update. Hum Mutat 33:887–894.
69. Kong X, Xing E, Zhuang T, Li P-K, Cheng X. 2021. Mechanistic Insights into the Allosteric Inhibition of Androgen Receptors by Binding Function 3 Antagonists from an Integrated Molecular Modeling Study. J Chem Inf Model 61:3477–3494.
70. Ekpenyong O, Cooper C, Ma J, Guy NC, Payan AN, Ban F, Cherkasov A, Cox MB, Liang D, Xie H. 2020. Bioanalytical Assay Development and Validation for the Pharmacokinetic Study of GMC1, a Novel FKBP52 Co-chaperone Inhibitor for Castration Resistant Prostate Cancer. Pharmaceuticals (Basel) 13.

CURRICULUM VITA

Isela Rodriguez was born and raised in Parral, Mexico. There, she earned her Bachelor of Science in Chemical Engineering in 2012. Later, she decided to pursue her Master of Science in Biotechnology in Chihuahua, Mexico, which she obtained in 2015. In 2016, she moved to El Paso, Texas to continue her education by pursuing a Doctor of Philosophy in Biological Sciences in The University of Texas at El Paso (UTEP).

During her studies, Isela learnt different chemical and biological techniques that allowed her to move forward in her career by being part of top-notch research laboratories. While studying at UTEP, she had the opportunity to develop and teach a research-based laboratory course from August 2017 to May 2023, where she was also in charge of maintaining chemicals and consumables in inventory and keeping health and safety compliance.

Furthermore, Isela participated in the publication of two review papers titled “A new pyridazinone exhibits potent cytotoxicity on human cancer cells via apoptosis and poly-ubiquitinated protein accumulation,” in the Journal of Cell Biology and Toxicology in December 2019, and “Structure and Function of the TPR-Domain Immunophilins FKBP51 and FKBP52 in Normal Physiology and Disease” in Journal of Cellular Biochemistry in April 2023. She also represented UTEP at different conferences such as Society for Basic Urological Research (SBUR) and the Midwest Stress Response and Molecular Chaperone Meeting (MWSM).

Currently, Isela is working for El Paso International Water Quality Laboratory as a Quality Control Supervisor.

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