2014-01-01

# A Comparative Approach to Assessing the Functional and Structural Characteristics of Human FKBP52 in the Regulation of Steroid Hormone Receptor Signaling Pathway

Diondra Crystal Harris
*University of Texas at El Paso*, DIONDRA.HARRIS@GMAIL.COM

# A COMPARATIVE APPROACH TO ASSESSING THE FUNCTIONAL AND STRUCTURAL CHARACTERISTICS OF HUMAN FKBP52 IN THE REGULATION OF STEROID HORMONE RECEPTOR SIGNALING PATHWAY

DIONDRA CRYSTAL HARRIS

Department of Biological Sciences

APPROVED:

Marc B. Cox, Ph.D., Chairman

Siddhardtha, Das Ph.D.

Igor C. Almeida, Ph.D.

Wen-Yee Lee, Ph.D.

Manuel Miranda-Arango, Ph.D.

Charles H. Ambler, Ph.D.
Dean of the Graduate School

# DEDICATION

I want to dedicate this project to my everlasting love, Jesus Christ. In Him alone is the source of all knowledge and wisdom in this universe. Thank you for allowing me to scratch the surface of you magnificent handy work that continues to confound the minds of men. I am in awe that you would use me. I pray that you will use this work to advance science and improve the quality of life for those afflicted with diseases. Thank you for trusting me with this amazing opportunity. All glory, praise, and honor to you, my Savior and King!

# A COMPARATIVE APPROACH TO ASSESSING THE FUNCTIONAL AND STRUCTURAL CHARACTERISTICS OF FKBP52 IN THE REGULATION OF STEROID HORMONE RECEPTOR SIGNALING PATHWAY

by

DIONDRA CRYSTAL HARRIS, B.S.

DISSERTATION

Presented to the Faculty of the Graduate School of
The University of Texas at El Paso
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
THE UNIVERSITY OF TEXAS AT EL PASO
August 2014

## ACKNOWLEDGEMENTS

I am thankful for the leadership and direction of my mentor, Dr. Marc Cox. Thank you so much for giving me a chance and allowing me to work in your lab. You have been instrumental in developing my scientific instinct and have always encouraged me to pursue my dreams. Thank you for being more than a mentor, but also a friend!

Special thanks go to my committee members: Dr. Igor Almeida, Dr. Siddhardtha Das, Dr. Manuel Miranda-Arango, Dr. Kristine Garza and Dr. Wen Yee Lee. Thank you for your encouragement and guidance.

I would like to thank the Border Biomedical Research Center's Biomolecule Analysis Core Facility, Tissue Culture Core Facility, and the DNA Analysis Core Facility, at the University of Texas at El Paso for the use of the instruments. I am also thankful for the guidance of Ana Betancourt for training me in DNA sequencing.

A huge thanks go to my past and present "lab family", particularly to Cheryl Samaniego, Yenni Garcia, Naihsuan Guy, Heather Balsiger, Johanny Tonos de Leon, Veronica Rowlett, Atanu Pal, Karen Olivares, Jeffery Sivils, and Marisa Salomon. A special thank you to my BFF, Cheryl Samaniego for always being there for me. Thank you Nia for always being real with me. Thank you Johanny for being the sunshine in the rain. And last but definitely not least, thank you to Yenni Garcia for all your help, Nour science dates, and encouragement. You girls have been amazing friends and I am thankful to God for you. Also thank you to the undergraduate students who helped me tremendously with my project. It was a joy mentoring you, I wish you all the best!

Lastly, I am grateful to my family and church family who have been the biggest support. To my Mother, Father, siblings and my love, Isaiah, you have always been my

biggest cheerleaders, words don't begin to express my gratitude to you all. Thank you for all your prayers, thank you for the emotional, financial, and spiritual support. To my church family at Northeast and Smyrna, thank you for your love and spiritual nourishment, especially the Santiago family. A special thank you to Dr. Renato Aguilera, Dr. Lee Aggison, Dr. Jacqueline Sylvester, and Dr. Chastity Bradford, who where instrumental in encouraging me to take this great leap of faith.

This work was supported by Research Initiative for Scientific Enhancement (RISE) Grant Number 5R25GM069621-08, The UTEP Department of Biological Sciences Teaching Assistantships (CLS), UTEP Grant Number 5G12RR008124 of the Border Biomedical Research Center (BBRC)/University of Texas at El Paso from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). This project was funded by the National Institutes of Health (NIH) Support of Competitive Research (SCORE) Grant 1SC1GM084863 (MBC), the National Institutes of Health (NIH) Supplement to the SCORE Grant 1SC1GM084863-02S1 (MBC), and the Cancer Prevention and Research Institution of Texas (CPRIT) Grant RP110444-P2 (MBC).

# ABSTRACT

The 52 kDa FK506 binding protein (h52) is a key positive regulator of Androgen Receptor (AR) in cellular and animal models and is an attractive target for the treatment of prostate cancer. Human FKBP52 is a known regulatory protein and co-chaperone that has been shown to play an important role in the regulation of the AR signaling pathway, and in the development of the male sexual phenotype. Cellular studies in mammalian and yeast cells reveal that FKBP52 is a positive regulator of AR, glucocorticoid receptor (GR), and progesterone receptor (PR), potentiating receptor-mediated gene expression up to 60-fold in some systems.

In targeting FKBP52, a thorough investigation and understanding of the structural elements that underlay its function is necessary. This permits a logical approach in targeting specific interaction motifs, such as those that exist between the AR and FKBP52. Here we use a cross-species comparative approach to analyze the mechanisms of potentiation and the functional difference between FKBP52 and *Danio rerio* (Zebra fish) FKBP52 (DrFKBP52). In this study we have taken advantage of this by comparing their differences to identify additional important domains and residues. Through this study we have identified the FK2 domain, a previously uncharacterized, non-functional domain, as playing a role FKBP52 activity. This observation dispels the notion that the FK1 domain is the sole regulatory domain, specifically the proline-rich loop. Though both have the proline-rich loop, its presence is not indicative of potentiation activity. A genetic selection screen generated in *Saccharomyces cerevisiae* for gain of potentiation activity, in a library of randomly mutated *DrFKBP*52 genes, identified two residues: position 111 in the FK1 domain and 157 in the FK2 domain as

being the critical residues for activation of receptor potentiation by DrFKBP52. In both the yeast model and mammalian cells, the *DrFKBP*52 mutation A111V, which is an adjacent residues downstream of the proline-rich loop, confer significant potentiation activity, whereas the same mutation introduced to FKBP52 only slightly elevates activity. Three dimensional crystal structure homology modeling by I-TASSER indicate that when alanine is replaced by valine at position 111 this change affect both the surface charge (to more neutral) and the hydrophobicity (to more hydrophobic) in vicinity. We believe this change induces an open conformation of the proline rich loop notch, allowing for sufficient surface area for AR interaction. A second residue in the FK2 region, T157R, also greatly influences potentiation. Moreover, the DFKBPr52: A111V _T157R double mutant potentiated hormone signaling as well as wild-type hFKBP52. Collectively these results suggest that specific residues in both FK1 and FK2 domain are critical for full activity and are involved in receptor interactions, which potentiates steroid hormone receptor activity. These newly identified domains and residues could possibly become targets for inhibitors as they could be key residues to specifically disrupt AR-FKBP52 association.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

## GLOSSARY OF KEY TERMS

17AAG – 17-N-Allylamino-17-demethoxygeldanamycin

52 KD 22RV1 – FKBP52 knock down 22RV1 cells

52 KO – FKBP52 knockout mice

52 KO MEF – FKBP52 knockout mouse embryonic fibroblasts

AAG – androstanediol glucuronide

ACS – American Cancer Society

ACTH- Adrenorcorticotrypic hormone

AD2 – autonomous activation domain 2

ADP-Adenosine diphosphate

ADT– androgen deprivation therapy

AF-2– Activation Function 2

AR – androgen receptor

ATCC – American Type Culture Collection

BAG-1 – BCL-2 athanogene-1

BCL-6 – B Cell lymphoma 6 protein

BF-1 – Binding Function 1

BF-3 – Binding Function 3

BPH – benign prostatic hyperplasia

ChIP – chromatin immunoprecipitation

CHIP – COOH terminus of the Hsp70-interacting protein

Co-IP – coimmunprecipitation

COX-2 – cyclooxygenase 2

CRPC – castration-resistant prostate cancer

CTD- C-terminal domain

CTE- Carboxyl terminal extension

CyP17-Cytochrome P450 17A1

CyP – cyclophilin

DBD – DNA Binding Domain

DHEA-Dehydroepianorosterone

DHT – 5α-dihydrotestosterone

DM – double mutant

DMEM – Dulbecco's modified eagle medium

DMSO – dimethyl sulfoxide

DOC- deoxycorticosterone

DrFKBP52- *Danio rerio* FK506 Binding protein 52

$EC_{50}$ – half maximal effective concentration

$EC_{20}$ – 20% maximal effective concentration

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

ELISA – enzyme-linked immuno sorbent assays

ER – estrogen receptor

FBS – fetal bovine serum

FDA- Food and Drug Adminstration

FEN1-Flap endonuclease 1

FKBP51– FK506 Binding Protein 51

FKBP52 – FK506 Binding Protein 52

FSH – follicle stimulating hormone

Foxo3a- Forkhead Box O3

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GR – glucocorticoid receptor

GST – glutathione-S-transferase

HDAC – histone deacetylase

HER2 – human epidermal growth factor receptor 2

HIP – Hsp interacting protein

HOP – Hsp organizing protein

HRE – hormone response element

HRPC – hormone refractory prostate cancer

HSF-1 – heat shock transcription factor 1

Hsp – heat shock protein

$IC_{50}$ – half maximal inhibitory concentration

ICAT – inhibitor to β-catenin and TCF-4

IGF-1 – insulin growth factor 1

Imp1 – importin B1

IRF-1 – interferon regulatory factor 1

KLK 2,3- Kallikrein related peptidase 3

LBD – ligand binding domain

LEF-1 – lymphocyte enhancement factor 1

LH – leutenizing hormone

LHRH – luteinizing hormone releasing hormone

LRH-1 – liver receptor homologue-1

MEF – mouse embryonic fibroblast

MEK-K – mitogen activated protein kinase

MEM/EBSS – minimal essential media/eagles essential salt solution

MPER – mammalian protein extraction reagent

MR – mineralocorticoid receptor

mTOR – mammalian target of rapamycin

NCoR- Nuclear receptor co-repressor 1

NCI – National Cancer Institute

NFKB- Nuclear factor Kappa light chain enhancer of activated B cells

NR – nuclear receptor

Nup62 – nucleoporin 62

PC- Prostate cancer

pCAF – p300/CBP-associated factor

PEST – proline, glutamic acid, serine, and threonine-rich

PI3K – phosphatidyl inositol-3'-kinase

PIH1 – protein interacting with Hsp90

PIN – prostatic intraepithelial neoplasia

PPAR- $\gamma$ – peroxisome proliferator activated receptor $\gamma$

PPIase – peptidyl prolyl cis-trans isomerase

PP5 – serine/threonine protein phosphatase 5

PR – progesterone receptor

PSA – prostate specific antigen

PTEN – phosphatase and tensin homolog

PVDF – polyvinylidene fluoride

RL – reticulocyte lysate

RLU – relative light units

RPMI – Roswell Park Memorial Institute

RT-PCR – real time PCR

SGT1 – small glutamine rich tetratricopeptide repeat protein 1

SHBG – sex hormone binding globulin

SHR – steroid hormone receptor

shRNA – short hairpin RNA

siRNA – small interfering RNA

Sp1-specificity protein 1

SRC 1, 2 – steroid receptor coactivator 1, 2

StAR- steroidogenic acute regulatory protein

SUMO – small ubiquitin like modifier

SWI/SNF – SWItch/sucrose non-fermentable

TAH1 – tetratricopeptide repeat-containing protein associated with Hsp90

TCF4 – ternary complex 4

TGF β – transforming growth factor β

TF- transcription factor

TIF-2 – transcriptional mediators/intermediary factor 2

Tip60 – HIV-Tat interacting protein

TMPRSS2 – human transmembrane protease serine 2

TPR – tetratricopeptide repeat

VEGF-2 – vascular endothelial growth factor 2

Wif1 – Wnt inhibitory factor 1

Wnt – wingless/int

Wt- wild type

# CHAPTER 1: INTRODUCTION

## 1.1 ANDROGEN RECEPTOR IN DISEASE

The Androgen receptor (AR) and androgens play a critical role in the regulation of male sexual development and physiological processes, specifically the development and maintenance of the male reproductive system. Given its crucial role in normal male physiology, deregulation of AR and androgen signaling pathways has been implemented in a variety of disorders and diseases such as: Androgen Insensitivity Syndrome (AIS), Spinal bulbar muscular atrophy (SBMA), and Prostate cancer (PCa). (38)

### 1.1.2 Androgen Insensitivity Syndrome (AIS)

The essential role of AR and androgens in male sexual physiology has been established through androgen insensitivity syndrome (AIS). This genetic disorder is a condition that results in the partial or complete inability of the target cells to respond to androgens. It is primarily caused by missense and nonsense AR mutations, resulting in amino acid substitution in the ligand binding domain (LBD) and DNA binding domain (DBD). Defective AR proteins are unable to bind androgens or androgen response elements (AREs). AR's inability to bind AREs inhibits the expression of androgen target genes in the body, dramatically affecting male sexual phenotype. Depending on the severity of receptor mutations, individuals with AIS can fall into two categories, complete AIS (CAIS) or partial AIS. Affected individuals have a Y chromosome, but still have mild to severe defects in external virilization. Mutation of part, or deletion of the entire AR gene leads to CAIS, resulting in a dysfunctional receptor protein that cannot respond to circulating androgens. CAIS can also be caused by an absence of androgens in the blood. Patients with CAIS display feminized external genitalia, intra-abdominal testes

and fail to develop secondary sex characteristics at puberty. Except for the absence of a uterus most CAIS individuals maintain all other female phenotype (44). The clinically relevant AR mutant, P723S, was identified in genital skin fibroblasts from a patient with complete androgen-insensitivity syndrome. This mutant was observed to have normal maximum androgen binding, but elevated equilibrium dissociation constants (45). Its activity was assessed in yeast and mammalian cells, and, as expected, the P723S mutant had minimal responsiveness to high amounts of dihydrotestosterone (DHT). Interestingly, in the presence of FKBP52, an Hsp90-associated co-chaperone, AR P723S matched the activity of wt-AR. Thus, the AR P723S mutant displays increased dependence on FKBP52 for function.

Partial AIS is also caused by AR mutations, which alter the structural conformation of AR and diminish AR responsiveness to androgens. The structural modification of the androgen receptor decreases the receptor's ability to bind hormones with high affinity, leading to reduced AR-mediated gene expression in target tissues. As a result, patients present with a number of physiological defects in male reproductive development (16). Interestingly, many of the defects seen in PAIS individuals correlate with those seen in *fkbp52*-deficient mice (6).

### 1.1.3 Spinal Bulbar Muscular Atrophy (SBMA)

Spinal bulbar muscular atrophy (SBMA), or Kennedy's disease, is an X-linked hereditary neurodegerative disease caused by expansion of CAG codon repeats. These repeats code for a polyglutamine (polyQ) tract at the amino terminus of the androgen receptor. Polymorphic repeat ranges from 11-35 CAGs in normal males and 37-65 CAGs in the SBMS, causing a dramatic impact on AR transactivation. Like native AR,

this ligand-dependent neurotoxic AR elicits effects through the normal steroid hormone receptor maturation cycle. Heat shock protein 90 (Hsp90) plays a critical role in AR maturation, contributing to receptor stability and the high affinity hormone binding conformation of the receptor. Once hormone is bound, the polyQ tract of AR is exposed, allowing either the association with other AR polyQ regions or abnormal conformational changes in AR. This leads to aggregates that are resistant to proteosomal degradation (46). Although transactivation is compromised, this doesn't result in a loss of transcriptional activity. Therefore, gain-of-neurotoxic effects caused by aggregates form intranuclear inclusion in motor neurons of the brain stem and spinal cord result in transcriptional deregulation. However, the exact molecular mechanism is still unclear. The physiological manifestations are muscle cramps, arm and leg weakness, difficulty speaking and swallowing and increased incidence of AIS. Treatment using anti-androgen such as Leuprorelin, Flutamide (reduce testosterone levels) and Dutasteride (5-α-reductase inhibitor) showed little to no improvement in SBMA clinical trails. Interestingly, the dutasteride study showed that higher levels of testosterone are associated with increased muscle strength and function (51). Hsp90 inhibitors (17-AAG and 17-DMAG) developed for the treatment of cancer have been studied for treatment in an SBMA mouse model. Both Hsp90 inhibitors were shown to decrease intranuclear aggregate formation, leading to an improvement in motor performance. Unfortunately, clinical trials for these inhibitors have not lasted more than 6 months due to toxicity including temporary blindness and liver toxicity (51). The lack of efficacy in anti-androgens and toxic Hsp90 inhibitors demonstrates the need to develop other therapeutic targets for this disease.

### 1.1.4 Prostate Cancer

Prostate cancer is a widely recognized disease that has become increasingly prevalent in American men. Except for non-melanoma skin cancer, prostate cancer is the most common diagnosed malignancy among men in the United States. It is the most common in males between the ages of 50-80 and is the second leading cause of cancer death among men of all races, killing approximately 1 out of 36 males a year (1, 2, 52, 53). Though PC affects all races it is most prevalent in the African American community, yet it has become more pervasive among Hispanics and Caucasians. The cause of PC is still unclear, however, scientists have found several causal links. Several rick factors include race, environment, age, obesity and heredity (49, 44).

Prostate cancer can be categorized into two groups, early stage and late stage. Early stage PC is usually characterized by the presence of abnormal cells and slow tumor growth that has not spread to surrounding tissues. At this stage treatment methods are more simplistic ranging from watchful waiting, surgery, radiation therapy, and androgen ablation therapy. In this case hormone therapy is the most commonly used since androgens induce AR gene activation and play a necessary role in maintaining the integrity of the prostate gland (2, 44, 50). Invariably, because of the evolutionary nature of cancer, the disease recurs with a phenotype resistant to further hormonal manipulations, thus beginning the catastrophic progression to late stage PC; also known as castration-resistant prostate cancer (CRPC). Late stage PC is a much more aggressive and complex cancer that is characterized as such because of the multifaceted avenues that PC utilizes for proliferation and survival. Nevertheless, researchers have slowly begun uncovering avenues by which CRPC mediates aberrant

AR activation in the presence of minimal to no androgens. It is important to note that in cell culture abnormal AR activation, in the absence of androgens, can be induced by one or more of the following pathways: mutations in the AR gene, permitting promiscuous ligand binding behavior, AR overexpression, hyperactive splice variants, and activation of the AR by growth factors and cytokines (54). In addition, stimulation of the cAMP-dependent protein kinase (PKA) pathway, interleukin-6 (IL-6), growth factors (55, 56, 57) expression of co-regulatory molecules, crosstalk with other intracellular signaling pathways (Wnt, NF-kB, bcl2/bcl-xL, PI3K/Akt) (58, 59, 60, 61) and bone-derived factors (64), also have roles in aberrant AR transactivation.

Although prostate tumor growth is independent of androgens, it still remains dependent on a functional AR for cancer cell growth and survival (65, 66). It is becoming increasingly evident that late stage PC tumors consist of multiple cells containing different AR mutations, which can be activated by one or more signaling pathways. Interestingly, spontaneously occurring AR mutations are observed after androgen ablation therapy. The mutations that seem to cause the most detrimental effects are near or in the ligand-binding domain (LBD) and N-terminal domain (NTD) and these include Q668P, I671F, M523V, and G524D (65, 62). *In vitro* studies determined that all mutants displayed a 2- to 4-fold increase in AR activity in response to dihydrotestosterone (DHT), estradiol, progesterone, adrenal androgens and AR antagonist (hydroflutamide). Structural studies showed a decrease in the hydrophobic surface of the LBD, suggesting an altered intra-domain communication between the NTD and LBD possibly mediating the activity of AR variants. Additionally, some mutants have the capacity to form intra-molecular non-covalent bonds, making it more probable

that conformational changes induce their transactivation properties (63, 65).

Researchers also implicated overexpression of AR at the mRNA and protein level in CRPC, suggesting that a role for transcriptional regulation of the AR gene could be one of the major causes of AR dysregulation. In most cases increased AR expression occurs without gene amplification and may be due to altered transcriptional regulation. Transcriptional co-activators play a key role in the expression of a wide variety of genes, including AR. These co-activators bind to DNA in a specific sequential manner and positively or negatively affect gene expression. The human AR is encoded by one gene located on the X-chromosome. Characterization of the human AR promoter region determined that it lacks a TATA-box and a CCAAT-box. Several transcription factors have been reported to be responsible for controlling AR transcription and promoting PC. Notable transcription factors (TF) that augment AR gene expression in PC are Sp1, Foxo3a, mutated p53, LEF1, NFkB, FEN1, Beta-catenin and cAMP (66, 67, 78). These nuclear proteins positively regulate AR transcription and promote growth of PC cells. Each uses a diverse pathway to exert effect. For example the Wnt-1/β-catenin pathway leads to activation of the LEF1 complex and increases AR transcription. Several studies have indicated that progression to CRPC is associated with AR overexpression, and that AR inhibition represses tumor growth in PC, even in CRPC (69, 70, 71, 72).

As researchers uncover the many facets of PC progression, the treatment paradigm continues to shift to more difficult and complex strategies in the recent years. Few effective therapies exist for the treatment of castration-resistant prostate cancer. Castration-resistant tumor growth is lethal and accounts for more 30,000 deaths in the

United States yearly. Median survival from the time of diagnosis for CRPC is between 2-3 years (75). Most therapies and treatments are directed towards androgens and the androgen receptor. The AR signaling pathway in CRPC can activate gene expression despite very low circulating levels of androgens. As aforementioned, AR signaling is critical for growth and survival of CRPC and a majority of castration resistant tumors still rely on AR activity for proliferation, making AR a rational target for CRCP. This fact is further corroborated by clinical efficacy of androgen synthesis inhibitors (CYP17) and the second-generation AR antagonist MDV3100. Second line hormonal therapy, CYP17 and MDV3100, were recently approved by the US FDA for treatment of CRPC for its cumulative improvement in patient population survival (74).

## 1.2 TREATMENTS

### 1.2.1 CYP17 Inhibitors: Abiraterone (Zytiga)

Cytochrome P450 17A1 is an enzyme found in the adrenal cortex that is encoded by the CYP17A1 gene. This monooxygenase is localized in the Endoplasmic reticulum, and catalyzes the synthesis of cholesterol, steroids, and other lipids. It is comprised of 17α-hydroxyase and 17,20-lyase activity and is a critical enzyme in the steroidgenic pathways that produce progestins, mineralocorticoids, glucocorticoids, androgens and estrogens. CYP17 inhibitors, abiraterone acetate, orteronel, and galeterone block the biosynthesis of androgens by inhibiting the CYP17A1 enzyme. However, only abiraterone (Zytige) received FDA approval in 2011 for the treatment of metastatic CRPC. Androgens are made from dehydroepiandrosterone (DHEA), which is derived from cholesterol. This four-step process begins when cholesterol is shuttled into the

mitochondria by steroidogenic acute regulatory protein (StAR). Cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme, Cytochrome P450. Pregnenolone then undergoes 17α-hydroxylation by microsomal Cytochrome P450c17. Finally, 17-OH pregnenolone is converted to DHEA by the 17,20 lyase activity of Cytochrome P450c17, which is where abiraterone acts to inhibit (75, 76).

Cytochrome P45017A1 is a key enzyme in cortisol synthesis via its 17α-hydroxylase activity and plays a central role in androgen biosynthesis with its 17,20-lyase activity catalyzing the conversion of 17-hydroxypregnenolone to the main androgen precursor DHEA (77). These therapeutic agents have the capacity to bind the enzyme's active site and mimic the substrate, thereby inhibiting enzyme activation. CYP17 inhibitors target steroidogenic pathways in testicular, adrenal/normal prostatic tissues and PC cells (78). However, side effects such as hypertension, edema, and hypokalemia were observed due to mineralocorticoid build up. This was counteracted through the combined administration with prednisone. It is believed that prednisone decreases the adverse effect of excess mineralocorticoid by suppressing ACTH release from the pituitary gland, thus disrupting its pathway (78). A 50% reduction in prostate specific antigen (PSA) levels is seen in approximately 60% of patients when abiraterone and prednisone are administered together. Clinical trials have determined that this drug is successful in eradicating the minimal amounts of androgens produced in the adrenal and intratumoral production of androgens (79).

### 1.2.2 MDV3100: Enzalutamide (Xtandi)

MDV3100 is a non-steroidal antagonist of AR that binds directly to the LBD of AR, inhibiting subsequent events that lead to gene expression and CRPC progression.

Castration-resistant prostate cancer is commonly associated with increased levels of AR gene expression, which can occur through AR gene amplification and AR slice variants (80) concluded that high AR levels alone were sufficient to cause the transition from hormone sensitive to hormone-resistant PC in mouse xenograft models, which were derived from men with hormone-refractory PC. The mechanisms of this transition are still unclear. However, this same study demonstrated that overexpression of AR propagated an imbalance in nuclear receptor NCoR, a co-repressor, recruitment to the PSA promoters (KLK3 and KLK2). This observation suggests that a modest change in the levels of AR protein can augment co-activator and/or co-repressor assembled to the promoter, thus affecting AR transactivation (81). With this said AR is the key functional component in the progression of CRCP. MDV3100 unlike its predecessor, bicalutamide, does not promote translocation of AR to the nucleus. Alternatively, MDV3100 prevents binding of AR to DNA, and co-activators, it binds the AR with higher affinity, down regulate PSA and TMPRSS2 and induces apoptosis in VCaP cells (71, 80). Since MDV3100 binds to the androgen receptor and blocks all subsequent actions, it can inhibit the growth of prostate cancer cells, which have failed standard hormonal therapies and even chemotherapies. The dramatic improvement in patient survival has made MDV3100 (Xtandi) the treatment of choice for CRPC.

As with most PC therapies, not all patients respond to drug treatment and most patients who initially respond to treatment will develop resistance. Although both of these drugs seem to be an answer to the CRPC dilemma, another paradigm shift occurs making novel therapies essential for the further management of PC and CRPC. Most cancers seem to have an evolutionary survival advantage, which allows tumors to thrive in the most obscure circumstances. This phenomenon is seen with the

development of abiraterone resistance in a castration-resistant VCaP xerograph mouse model. Relapse is caused by up-regulated CYP17 expression, suggesting that abiraterone exerts selection pressure on cell survival and up-regulates intratumoral CYP17 expression (83) and/or induces AR and AR splice variants that confer ligand-independent AR transactivation. MDV3100 also succumbs to the same fate, MDV3100 resistance. Mechanisms of resistance are still unclear, but may be due to AR mutations that confer drug resistance (84), splice variants that lead to constitutive ligand-independent AR activation, or alternative signaling pathways. Activation of the PI3K signaling pathway by the loss of PTEN has been shown to impair AR gene expression leading to decreased dependence on AR signaling. PTEN-null mouse models treated with MDV3100 demonstrated 25- to 50-fold decreases in cytoplasmic mRNA levels of AR dependent genes with increased phosphorylation of PI3K pathways such as AKT. This was suppressed by concurrent treatment with a PI3K inhibitor. Transcriptome analyses reveled that activation of the PI3K pathway is associated with repressed androgen signaling *in vivo* and *in vitro,* and may be responsible for the castration-resistant phenotype observed in MDV3100 resistance (85). Cumulatively, the data indicate that AR and PI3K are cross-regulated by reciprocal feedback, thus, inhibition of one activates the other, maintaining tumor cell survival. Therapeutic drugs, although successful, eventually fail due to drug resistance, which necessitates the continued development of novel therapies that can regulate prostate cancer signaling pathways.

### 1.2.3 Hsp90 Inhibitors:17-AAG (Tanespimycin) and 17-DMAG (Alvespimycin)

A large number of oncogenic proteins are overexpressed in cancer cells and inhibiting the function of these proteins is also essential in controlling the progression of

cancer. Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone that regulates the folding of over 300 nascent client proteins (86). This evolutionary conserved chaperone maintains cell homeostasis in proteotoxic stress conditions by binding unstable proteins and prevents protein degradation and aggregation. While overexpression occurs for many oncoproteins, mutations accumulate facilitating cancer cell survival through Hsp90 stabilizing events.

Hsp90 also plays a pivotal role in the integrity of the steroid hormone receptors, and its association is necessary for hormone receptors to achieve the high affinity hormone binding conformation. Because Hsp90 is vital for AR structural integrity, it too has been identified as a possible therapeutic target in CRPC. Hsp90 dynamic chaperoning occurs when ATP binds to the N-terminal domain, and ATP hydrolysis by Hsp90 facilitates a conformational cycle that is essential for its chaperone activity. The Hsp90 inhibitor, geldanamycin, binds directly to the N-terminal ATP-binding pocket, thereby preventing ATP binding, and leading to a loss in chaperone activity and subsequent client protein degradation (87). A geldanamycin derivative, 17AAG, has recently entered clinical trails for the treatment of cancer. 17AAG is an ATP-antagonist that blocks p23 association with Hsp90, which destabilizes interactions with client proteins, and induces proteasomal degradation. Since the Hsp90 oncogenic client protein repertoire has been discovered, these inhibitors may combat oncogene switching, which seems to be a critical mechanism of cancer cell survival (82). Targeting chaperones and/or co-chaperones that regulate AR activity is another avenue to combat the ever-evolving CRPC. However, understanding the unique environment in which metastatic tumors thrive at the consequence of its host remains an area of much

needed research.

## 1.3 ANDROGEN RECEPTOR SIGNALING PATHWAY

### 1.3.1 Androgen Receptor

The androgen receptor (AR) belongs to an important class of transcription factors that regulate a diverse number of physiological functions, including control of embryonic development, cell differentiation and homeostasis. This sub-family of steroid hormone receptors includes glucocorticoid receptor (GR), progesterone receptor (PR), estrogen receptor (ER) and mineralocorticoid receptor (MR). The AR gene is located on chromosome Xq11-12 and spans 180 kD of DNA with eight known exons, encoding for a protein that is structurally and functionally similar to other nuclear receptors. It exists in two isoforms, AR-A and AR-B that are created by splice variants. AR-B is the full-length form, 110kDa and AR-A is the truncated form, 87kDa, however, other truncated variants are present in prostate cancer tissues. Here, I will focus on AR-B, which is found predominantly in the cytoplasm and nucleus of the cell. AR-B functions as a dimer in the nucleus to regulate the transcription of target genes in a ligand-responsive manner. Its ligands, androgens, are sex hormones that belong to the steroid hormone family. In gestation, testicular androgens are a necessary differentiation process that leads to primary sex characteristics of males. AR has the greatest binding affinity towards 5α-dihydrotestosterone (DHT) followed by testoterone, which dissociates from the receptor more rapidly than DHT. The prototypical AR protein contains several functional domains. An N-terminal transactivation domain (NTD), a highly conserved DNA-binding domain (DBD) encoded by exons 2 and 3, a hinge region and a carboxyl

terminal ligand binding domain (LBD) encoded by exons 4 to 8, as seen in Figure 1.1 (89,90 92).

The $NH_2$-terminal domain (NTD), encoded by exon 1, constitutes more than 60% of the AR protein, and is the least homologous in sequence between the steroid hormone receptors. The NTD is a major transactivation domain, is structurally flexible, and functions in multiple protein–protein interactions with co-regulatory proteins and other components of the general transcription machinery. This region contains several homopolymeric amino acid stretches, a polyglutamine region, nine proline residues and 24 glycine residues. It is thought that the polyglutimine and the nine prolines are important in transcriptional regulation via its protein-protein interaction with other co-regulators. AR transactivation is determined almost exclusively by the NTD (94). The NTD interacts with the C-terminal domain (CTD) through a FQNLF motif, in an intramolecular head-to-tail interaction, prompting gene expression (19). Deletion studies show that there are several isolated transcriptional activation domains within the AR NTD that act on transcription differently depending on cell lines and the use of different promoters. These domains are known as, smaller activation function 1 and 5 (AF1 and AF5) (95). The AF1 domain is located between residues 101-370 and is required for full ligand activated transcriptional activity. The AF1 is an activation site and is thought to be the major domain responsible for mediating AR transcriptional activity. AR, in addition to GR, is unique in that most of its activities are mediated by the constitutive activity of AF1 (96). AR AF1 interacts with the large subunit of the general transcription factor TFIIF, termed RAP74, and the steroid receptor co-activator 1 (SRC-1) (97). Co-activators alter chromosome structure and can enhance or repress transcription activity by changing

the accessibility of the specified DNA region to the general transcription machinery. AF5 is located between residues 360-485 and is responsible for the constitutive activity (activity without bound ligand). AF5 is comprised of three amino acid stretches: proline stretches (371-378), an alanine stretch (397-401) and a glycine stretch (448-463). Although very little is known about AF5, studies indicate that deletion of the ligand binding domain, which results in the loss of AF1, induces the use of AF5. It has been determined that elongated Gln repeats of more than 40 residues in this region is associated with Kennedy's disease (99). Furthermore, AR contains the small ubiquitination like modifier (SUMO)ylation sites K386 and K520, that are thought to repress gene transcription (98).

The DBD is a cysteine-rich region of AR made up of two zinc binding motifs and has a loosely structured carboxyl terminal extension (CTE). This region is the most conserved region among steroid hormone receptors (SHRs), displaying approximately 80% amino acid identity with MR, PR and GR, and is critical for the folding and function of the domain. The first zinc finger interacts with the major grooves of the DNA termed the P-box and is the recognition helix that interacts with transcriptional enhancers. The second zinc finger interacts with the D-box, which is highly basic and stabilizes DNA-receptor interaction by contact with the DNA phosphate backbone and mediates accurate receptor dimerization. Studies indicate that the receptor may not directly bind to DNA, but piggyback onto other DNA-binging proteins such as the AP-1 heterodimer components Fos and Jun. The CTE is also essential for response element recognition and high affinity binding, specifically of the AR (100, 101).

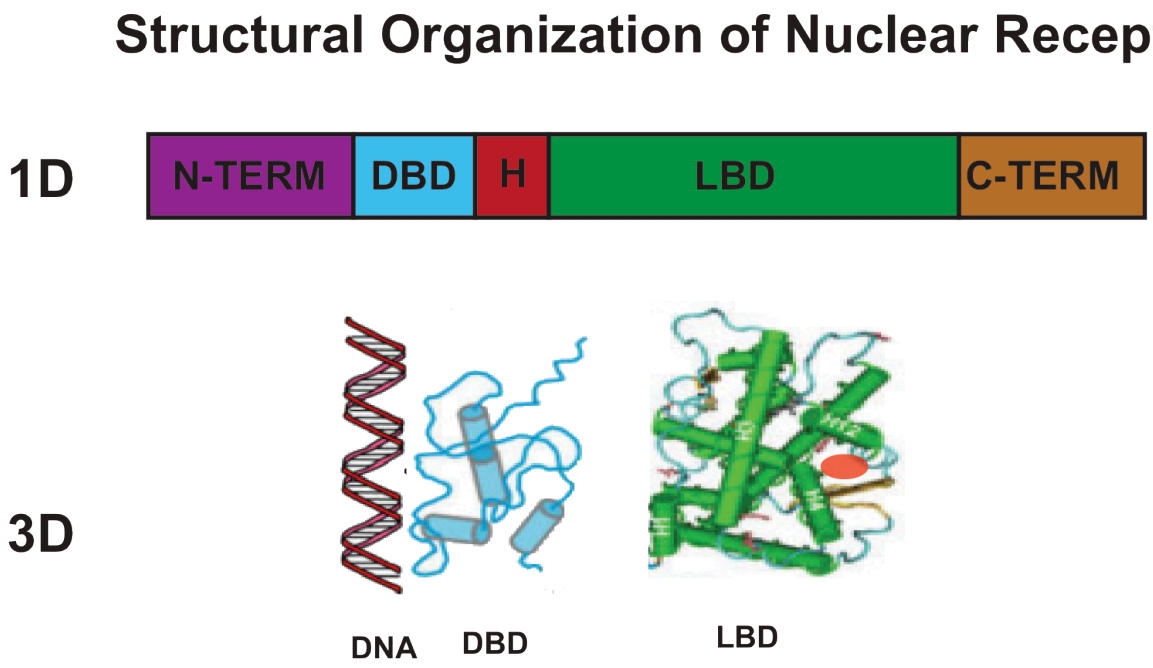



**Figure 1.1: Domains of Steroid Hormone Receptors**

Major domains are identified in color. The 3D structures show an illustration of the DBD interacting with DNA and LBD interacting with ligand.

The hinge region is a flexible linker located between the DBD and the LBD. It is poorly conserved and contains a nuclear localization signal and export sequence, which is critical for the transfer of AR from the cytoplasm to its site of action in the nucleus (21). The hinge region also undergoes various post-translational modifications including acetylation and phosphorylation. Acetylation of AR by p300/CBP-associated factor and HIV-Tat interacting protein (Tip60) represses transcriptional activity through decreasing activation of the receptor, nuclear transactivation and cofactor recruitment to the promoter. Mitogen Activated Protein Kinase (MEK-K) also phosphorylates AR, which assists in translocation (102, 19).

Finally the ligand-binding domain (LBD) shares a 50% identity with GR, MR, and PR. The LBD functions to bind androgens with high affinity, it's an interaction surface for Hsp90, and dimerization of the receptor. When ligand binds, it induces a conformational change of the last helix, which stimulates the transformation of a hydrophobic region termed activation function 2 (AF2) for co-activator binding (103). Several co-activators bind to this surface via the conserved LXXLL or FXXLF binding motifs to enhance the transcriptional activity of the receptor (105, 106. The LBD has an internal binding site that is comprised of 18 crucial residues located in helices 3, 5, and 11, known as Binding Function 1 (BF1). Helix 12 operates as a gate on the ligand binding domain; it opens to permit ligand binding and closes once ligand has bound (104). Recently another co-activator binding site has been identified that is adjacent to AF2. It is thought that this surface could be the site of protein-protein interaction for AR regulatory binding proteins and has been termed binding function 3 (BF3) (103). Thus, the androgen receptor is very tightly regulated, and mutations that comprise receptor structure and

function can lead to the development of numerous diseases, including prostate cancer. (15,16,17).

### 1.3.2 Steroid Hormone Receptor Complex

Steroid hormones (SH) are lipid-signaling molecules that are synthesized in the adrenal cortex and gonads from cholesterol. Cholesterol is a soft, waxy, fatty compound that is produced in the liver and further synthesized in mitochondria and smooth ER of target organs. It is an important nutrient that is essential in the formation and maintenance of cell membranes, and in the production of sex hormones. All steroid hormones are derived from cholesterol and regulate a large number of physiological processes in target cells. There are five major groups of steroid hormones: estradiol, progestins, androgens, glucocorticoids, and mineralocorticoids. All steroids share a carbon skeleton composed of four fused rings, a 3 six-member ring and 1 five-member ring, at position C-17 there is a substitution that varies from hormone to hormone, depending on desired function. ( 44, 108)

In order for steroidogenesis to occur the appropriate enzymes are necessary. Enzymatic activities involved in androgen synthesis in the testis are mainly localized in Leydig cells. Testosterone production is controlled by Lutenizing hormone (LH), which regulates the expression of the enzyme 17-β hydroxysteroid dehydrogenase and converts androstenedione to testosterone. Once cholesterol enters the Leydig cells and is stimulated by LH the cholesterol is modified by an enzymatic cascade located in the mitochondria and smooth endoplasmic reticulum (sER). Steroidgenic acute regulatory proteins transport the cholesterol from the external membrane to the internal membrane of the mitochondria where steroid hormone (SH) modification begins. First,

CYP11A1/Demolase converts cholesterol to Pregnenolone. Pregnenolone can then diverge into 3 different pathways depending on desired steroid hormone. The 2nd step occurs in the sER in the case of androgens, pregnenolone is converted into 17-hydroxypregnenolone by CYP17 or progesterone by 3-β-hydroxysteroid dehydrogenase (3-β HSB). 3-β HSB is the only enzyme within this pathway that is not apart of the cytochrome P450 family. 17-hydroxypregnenolone is further reduced by CYP17 to dehydroeplandrosterone, which is then converted to Androstenedione by 3-β HSB. Lastly, 17-β HSB converts androstenedione into testosterone, which is secreted into the bloodstream where they are bound to carrier proteins, called sex hormone-binding globulins. Androgens are lipophilic molecules that enter the target cells by simple diffusion (8). Once in the cytosol, steroid hormones are cleaved by 5α-reductase and other molecular modifications such as phosphorylation, which make them suitable ligands to bind their appropriate steroid hormone receptor within the Steroid Hormone Receptor Complex (SHRC) (8,9).

SHRs are key mediators of steroid hormone ligand action. All SHRs are transcription factors that bind DNA and turn on gene expression, and are mainly found, in the absence of hormone, in the cytoplasm in complex with chaperone proteins. These receptors are constantly undergoing dynamic nucleocytoplasmic restructuring by forming large oligomeric structures with one or more chaperones, co-chaperones and/or co-activators known as regulatory proteins. Figure 1.2 illustrates the classic GR maturation process. There are 3 stages of receptor maturation including early, intermediate, and late folding events.

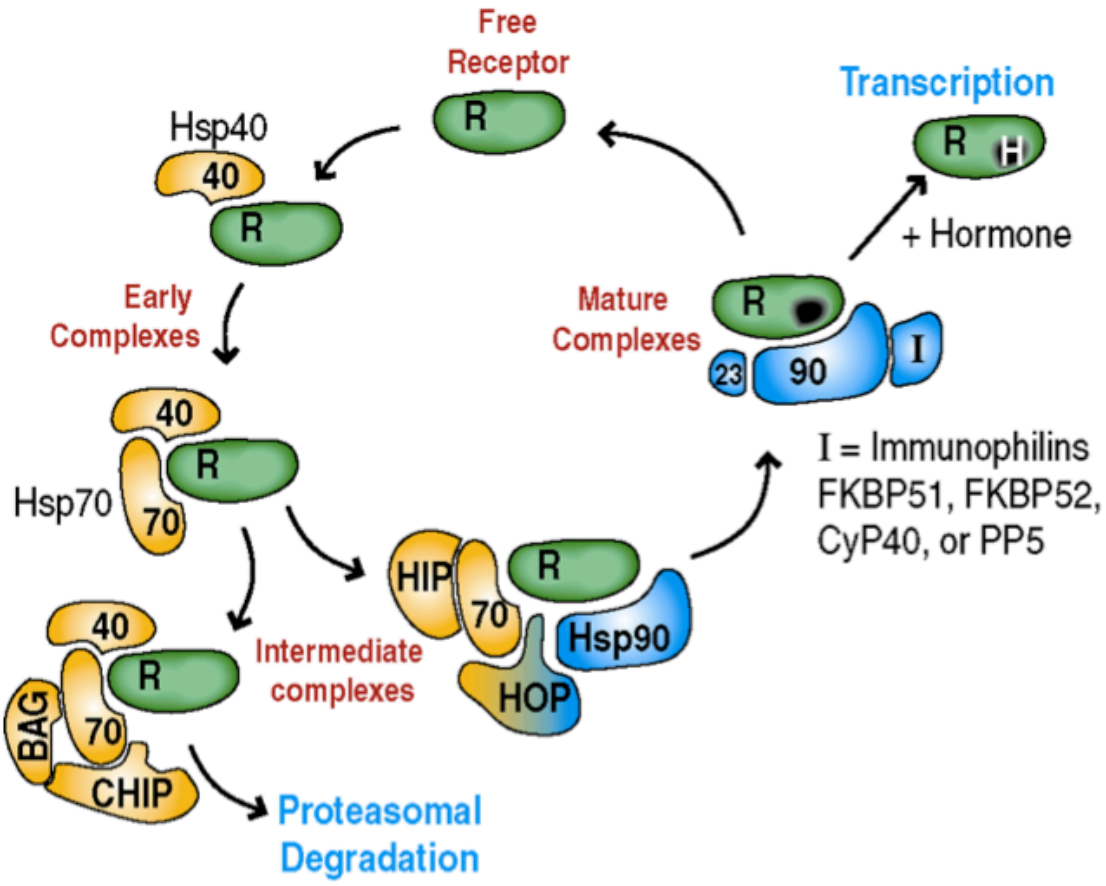



**Figure 1.2: Steroid Hormone Receptor Maturation Cycle**

Figure 1.2 depicts a General Illustration of the Chaperone Mediated Receptor Maturation Complex.

At every stage the chaperones and co-chaperones associate and disassociate with the receptor as it progresses to its high affinity hormone binding conformation. The association of Hsp40 to the naïve receptor LBD characterizes the early complex, which is a prerequisite for Hsp70 recruitment. Hsp40 initiates Hsp70's ATPase activity, permitting Hsp70 to form a direct interaction with the LBD. The intermediate complex allows the association of Hsp Interacting Protein (HIP) and Hsp Organizing Protein (HOP) or chaperone cofactors such as BAG-1 and CHIP with hsp70. When steroid hormone receptors are improperly folded they are tagged with ubiqitin ligase (CHIP) and are tagged for the proteasomal degradation pathway. HIP increases the stability of the ADP-bound form of Hsp70 and Hop is a tetratricopeptide repeat (TPR)-containing protein that acts as an adaptor protein promoting the transition from hsp70 to the hsp90 dimer. Once hop recruits the Hsp90 dimer, hsp70, Hip, and hop dissociate. At the mature stage Hsp90 recruits the p23 cochaperone and one of a family of immunophilin proteins (FKBP52, FKBP51, PP5 and Cyp40), resulting in a stable complex that is capable of high affinity hormone binding (10,11). Hsp90 contains a nucleotide-binding domain that acts as an ATP/ADP switch domain, regulating hsp90 confirmation. It shows a higher affinity to hydrophobic substrates when bound by ADP, while the p23 bound form has more efficient chaperoning properties. p23 stabilizes the receptor-hsp90 heterocomplex in an ATP-bound manner and is highly conserved between animals, plants, and yeast. SHR localization to the nucleus is signified by a nuclear localization signal (NLS) found in the hinge region and DBD of the steroid hormone receptor. This region of the receptor is exposed once mature complex dissociates and undergoes a conformational change releasing the Hsps. The receptor dimerizes, rapidly

translocates to the nucleus and binds the appropriate hormone response element (HRE). However, experimental evidence suggest another model for nuclear shuttling, implicating the mature complex in nuclear translocation. It is thought that GR-Hsp90/p23/immunophilin transports the mature complex to the nucleus and also can interact with the nuclear pore proteins, importin-β and Nup62 (107). Furthermore, hormone binding of the SHR dimer modifies the secondary and tertiary structure of the chromatin, which attracts other transcriptional cofactors such as p160, and p/CAF (8,12). These cofactors facilitate further chromatin remodeling, making it conducive for active gene transcription, thus, regulating the expression of hormone responsive genes. Thus, disruption of SHR folding indirectly through changes in the components of the SHRC could lead to devastating effects on the human reproductive system (infertility), interfere with proper sexual development (androgen insensitivity) and contribute to various cancers (breast and prostate cancer) (13,14,15).

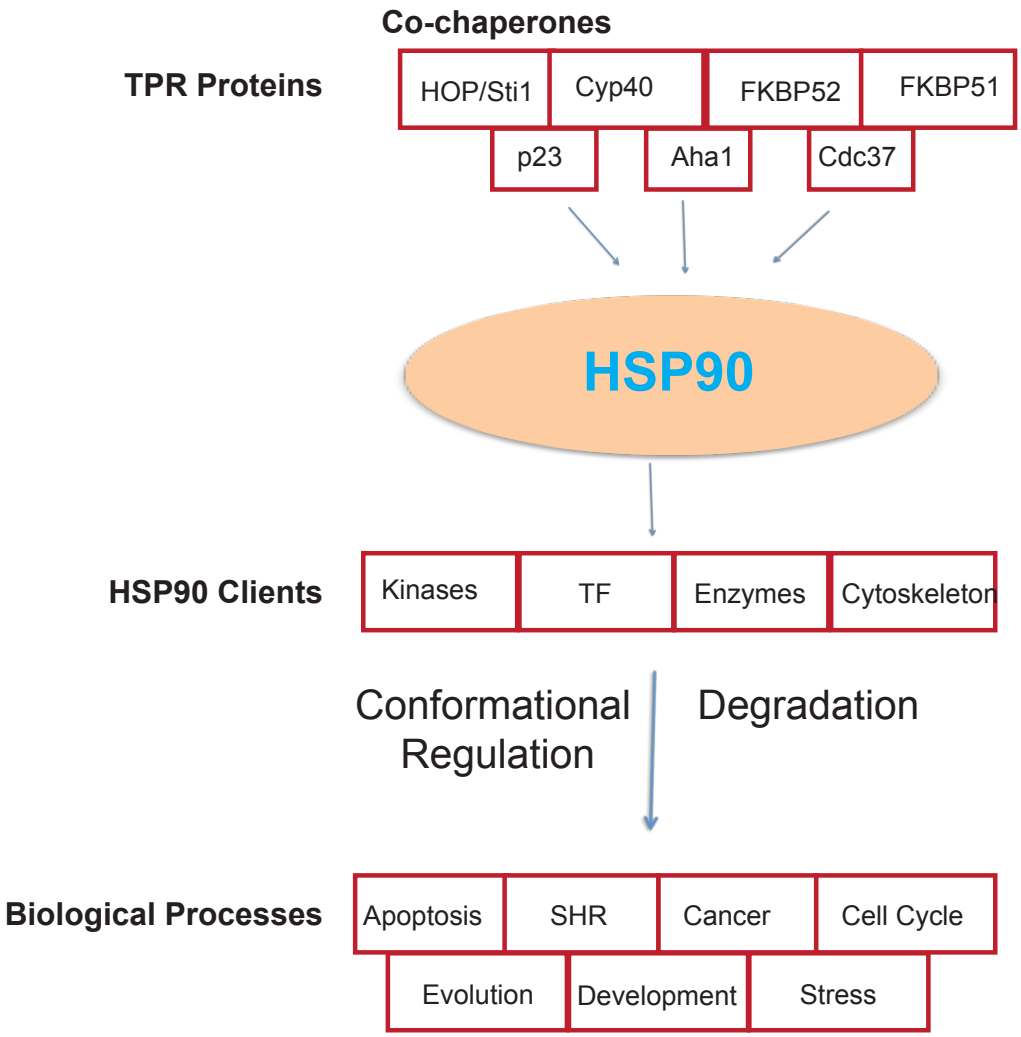



**Figure 1.3: Hsp90 Client Proteins.**

Illustration depicts various HSP90 Co-chaperone binding partners, varies Client protein that rely on HSP90 activity and the multiple biological pathways HSP90 is involved.

### 1.3.3 FK506 Binding Proteins

Immunophilins are a functionally diverse group of co-chaperones that are known for their ability to bind immusuppressive drugs through the peptidyl-prolyl cis-trans isomerase (PPIase) domain. Immunophilins are known for their PPIase enzymatic activity, although, it is now known that PPIase activity is not involved in immune responses or chaperone activity (108). PPIase enzymatic activity catalyzes the cis/trans isomerization of peptidyl-prolyl, which affects the backbone arrangement of the target protein (17, 18). Immunophilins can be separated into two major classes based on their ability to bind immunosuppressive drugs. The first class is cyclophilins, which bind cyclosporine and the second is FK506-binding proteins, which bind FK506, FK1706, and rapamycin (18). FK506-binding proteins are the only class of proteins that will be further discussed here. FK506-binding proteins (FKBPs) are a unique class of immunophillins that perform many cellular functions including protein folding, regulation of cytokines, nucleic acid binding, histone assembly, and modulation of apoptosis. There are 15 FKBPs in humans and most of them possess numerous functional motifs and FKBP domains such as: nucleic acid binding regions, TPR domains, calcium-binding domains, transmembrane domains, nuclear localization signals, and endoplasmic reticulum signal sequences. The diversity in these proteins enables them to perform a wide variety of cellular functions, including protein folding. FKBP12 is the most comprehensively studied and smallest (108 aa) of the FKBPs, containing only one PPIase domain (18). FKBP12 is an ubiquitous, abundant protein that is a receptor for rapamycin, which elicits its effects by binding to and inhibiting intracellular calcium

release channels and the transforming growth factor β (TGF-β) type I receptor (21). Many of the larger FKBPs are more complex, containing multiple protein-protein interaction domains, and are involved in the regulation of high molecular weight complexes including the SHRC.

### 1.3.4 FKBP51 and FKBP52

Human FKBP52 and FKBP51 are two large immunophilins that promote and inhibit, respectively, the activity of AR, GR, and PR. FKBP51 and FKBP52 are evolutionarily related Hsp90-binding co-chaperones, yet they have unique interaction with Hsp90 and Hsp90 client proteins. Although Hsp90 binds many client proteins within the SHRC, it recruits specific co-chaperones based on which client protein is bound. Figure 3 shows an illustration of Hsp90's many client proteins and TPR containing proteins that participate and support Hsp90-client interactions. FKBP51 and FBP52 also have chaperoning activity that is independent of both Hsp90 and PPIase activity (28). Hsp90 is the most abundant cytoplasmic chaperone involved in the stress response and has a critical role in stabilizing proteins. Studies have shown that Hsp90 association is required for the normal activity of SHRs, and in SHRCs it is typically bound to one of the large immunophilin co-chaperones including FKBP51, FKBP52, Cyclophilin 40 (CyP40), or protein phosphatase 5 (PP5) (25). FKBP51 and FKBP52 are composed of three distinct domains and are highly similar in sequence and structure as seen in figure 1. 4. The FK1 domain consists of a functional PPIase active site, while FK2 has a similar structure but differs at several amino acids that are critical for PPIase activity and drug binding (22).

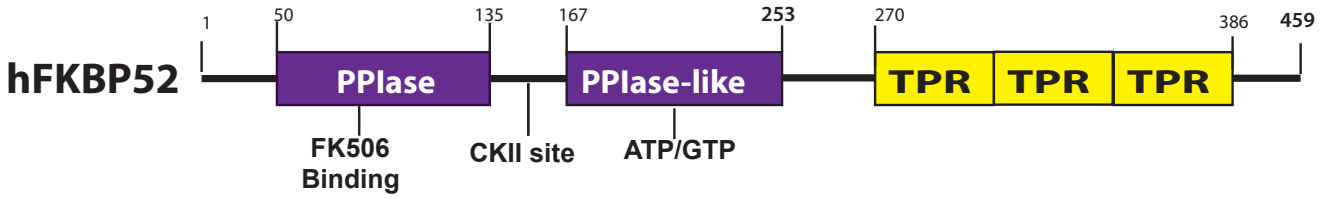



**Figure 1.4: Functional Domains of Human FKBP52.**

FKBP52 has 3 distinct domains:FK1 domain: PPIase active site, FK2:PPIase like domain, a TPR: Hsp90 interaction site.

The FK2 domain also contains a consensus ATP/GTP-binding sequence between amino acid 199 and 222. The region between FK1 and FK2 contains a hinge linker region (FK loop) that is stabilized by extensive hydrogen bonds and hydrophobic interaction on both FKBP51 and FKBP52. There is also a TEED functional sequence between FK1 and FK2 that is phosphorylated by casein kinase 2, thereby preventing binding of FKBP52 to Hsp90. Studies indicate that phosphorylation at this site decreases FKBP52 potentiation of receptor activity. The corresponding sequence in FKBP51 is FED, which lacks the consensus phosphorylation site. It is believed that this mutation impacts the ability of FKBP52's linker region to form hydrogen bonds properly (6, 40). In addition, the FK2 domain in FKBP51 is thought to be brought into close proximity to the receptor by Hsp90 for direct interaction with the receptor, and is required for preferred binding to Hsp90-GR complex (29).

The third domain is composed of three TPR domains that interact with the C-terminal region of Hsp90 (24). The TPR motif contains six helixes, with a seventh helix that extends beyond the core TPR domain. In FKBP51, the TPR domain projects at an angle from the rest of the protein, but in FKBP52, the TPR domain extends linearly with the rest of the protein. Hsp90 binding is mediated by electrostatic interactions between the TPR motif and the highly conserved MEEVD motif in the extreme C-terminus of Hsp90,,and by hydrophobic interactions with residues upstream of MEEVD. Studies have determined that the hydrophobic interaction with the peptide is critical for specificity (25, 26). FKBP51 exhibits ionic bonding between the FK2 and TPR domains, whereas FKBP52 lacks these bonds and is therefore more flexible in nature (108).

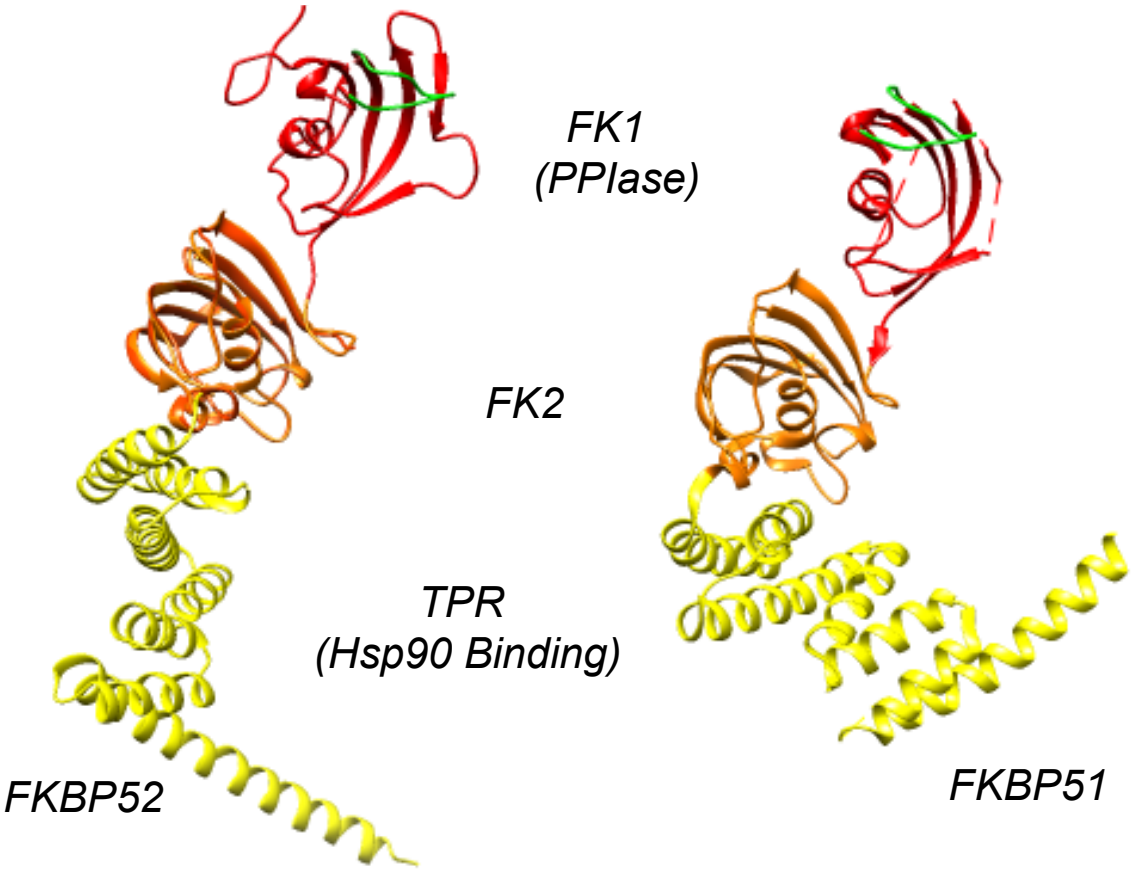



**Figure 1.5: X-ray Crystallographic Structures of FKBP51 and FKBP52.**

The three-dimensional structure of the composite of two partial structures for human FKBP52 (protein databank: 1Q1C and 1P5Q) and the structure of human FKBP51 (protein databank: 1KT0) are shown in ribbon format. The important functional regions FK1 (red), FK2 (orange), TPR (yellow), and the proline loop (Green), are illustrated. The figure was created using UCSF Chimera version 1.5.

In addition molecular studies indicate that FKBP51 and FKBP52 may augment receptors by different mechanisms. They both bind Hsp90 differently, FKBP52 only interacts through the TPR domain, but FKBP51 requires additional sequences up and downstream of the TPR domain (109). The forth domain, unique to FKBP52, is a putative binding site for calmodulin. While FKBP52 does not associate with calcineurin, an FKBP52 K121L mutant has high affinity for calcineurin (27).

Although similar in structure and sequence, FKBP51 and FKBP52 functionally diverged in their ability to regulate steroid hormone receptor activity. Both *in vitro* and *in vivo* studies show that FKBP52 positively regulates AR, GR, and PR signaling pathways (28, 30, 31). While FKBP51 does not inhibit hormone receptor activity in yeast, it can block the potentiation mediated by FKBP52 when co-expressed (6). FKBP52 has a distinct functional influence on the AR and PR signaling pathways, is critical for proper development of vital male reproductive organs, and may play a critical role in male and female fertility.

The physiological importance of FKBP52 was demonstrated through the use of FKBP52 knockout mice (52KO). The 52KO male mice had reproductive phenotypes including infertility, abnormal external virilization, ambiguous external genitalia, and dysgenic seminal vesicles and prostate, which can all be attributed to the loss of steroid receptor activity. The physiological/developmental abnormalities of the 52KO mice parallel those of human pathologies seen in AIS, which is linked to the desensitization of AR. Studies done in New World Primate also demonstrate that reduced expression of FKBP52 is associated with androgen insensitivity syndrome. Liquid chromatography-tandem mass spectrometry analysis found that serum derived from squirrel monkeys

had very high levels of androgenic hormones during breeding and non-breeding seasons when compared to humans. Dihydrotestosterone (DHT) levels in squirrel monkeys can reach 4.8 +/- 0.7 verses 0.33 +/- 0.01 in humans. Western blot analysis demonstrated that FKBP51 is higher and FKBP52 is lower in squirrel monkeys, which was thought to be a probable cause of reduced AR activity. Squirrel Monkey FKBP51 had no effect on DHT-stimulated AR activity. However, the overexpression of FKBP52 resulted in a significant increase in reporter activity. Therefore, researchers concluded that low androgen responsiveness in squirrel monkeys is the result of low FKBP52 expression (32).

Although the female 52KO mice had no morphological abnormalities, they were completely infertile. This infertility was due to maternal failure of embryonic implantation and decidualization; both processes are stimulated by, and dependent on, progesterone receptor activity. Interestingly, no defects in male and female physiology were seen in FKBP51 knockout mice, suggesting that FKBP51 is not necessary for AR activity in vivo. (33). However, the loss of both FKBP51 and FKBP52 in mice results in embryonic lethality. Thus, FKBP52 plays an indispensable physiological role, which cannot be substituted by the presence of other homologous proteins (28).

Additional cellular and yeast studies continue to substantiate FKBP52's role in AR signaling. FKBP52 is thought to have various functional roles within the AR signaling pathway. There is evidence that FKBP52 enhances receptor transcriptional activity by increasing the receptor's affinity for androgen, mediated by conformational changes within the AR ligand-binding domain (23,24). Studies also suggest that FKBP52 may influence the receptor's movement to the nucleus because FKBP52 is a regulator of

microtubules and associates with the dynein motor protein, thereby regulating SHR translocation to the nucleus (34, 35). This is still unclear as another study (Baulieu) has shown that FKBP52 promotes microtubule disassembly, which is a direct contradiction to this theory. FKBP52 does interact with dynein and this may be a functional interaction that is important for sperm motility (flagellar motor proteins) as the 52KO mice have defects in sperm motility. However, this may have nothing to do with SHRs as sperm motility is not an androgen-dependent process. It has also been noted that FKBP52 is not required for AR to be competent for hormone binding (28,6). Though FKBP52 is not required for hormone binding, research has consistently shown that FKBP52 has the ability to up-regulate AR signaling 20-fold, in comparison to other closely related immunophilins such as FKBP51, Cyp40, and PP5 (6). This strongly supports a role for FKBP52 in reproductive development in both male and female mice. However, the mechanism by which FKBP52 modulates receptor expression is not well understood. The evidence discussed above, and other recent findings implicating a role for FKBP52 in androgen insensitivity, infertility, and prostate cancer, has lead researchers on a quest to better understand the significance of FKBP52 within the steroid hormone receptor signaling pathway (8,11,28,36).

FKBP51 was discovered as a component of chicken PR complexes and is an Hsp90 co- chaperone within the SHRC. FKBP51 inhibitory effects were initially characterized in New World primates. The insensitivity to glucocorticoids and progestins in New World primates has been linked to the overexpression of FKBP51. Human FKBP51 inhibits both receptors' function. Mapping studies have shown that amino acid changes increase inhibitory actions of squirrel monkey FKBP51 (32).

FKBP51 has not been fully characterized in the SHRC, but *in vitro* studies show that FKBP51 inhibits SHR function through competition with FKBP52 for binding Hsp90. On the other hand, other Hsp90-binding TPR proteins do not block FKBP52 action as compared to FKBP51, so FKBP51 inhibition of SHR activity may not be through simple competitive inhibition of FKBP52 regulation.

Researchers have established that there are distinct functional differences between FKBP51 and FKBP52 within the SHRC. However, the underlying structural and functional relationship between the two proteins has only recently been thoroughly investigated. Sequence analysis of FKBP51 and FKBP52 shows that FKBP51 has 70% amino acid sequence similarity to FKBP52. Crystal structure analysis also shows an almost identical conformation within the structural domains. An additional study utilized Threading/ASSEmbly/Refinement approach (a protein tertiary structure prediction algorithm) to analyze FKBP51 and FKBP52 structure. This study showed that they possess the same structural arrangement consisting of two FKBP domains and 3-4 pairs of helical TPR motifs, with the FKBP domains rotating approximately 90 degrees about a 10-residue loop. Structural analysis also determined that there is an apparent distinction in FKBP51 and FKBP52 domains, particularly within the FK2 and TPR domains. The FKBP52 TPR domains are aligned in a more linear orientation, while the TPR motifis of FKBP51 are packed more closely to the FK2 domains, giving it a kinked confirmation, which could account for FKBP51's higher affinity for hsp90 binding (18, 28). FKBP51 contains a side chain that forms a salt bridge between the FK2 and TPR domains that stabilizes the interaction between its domains. Though FKBP52 lacks this salt bridge, it could contribute to the flexible structure of FKBP52, which may be

responsible for it interactions with more SHRs (38, 39).

FKBP52 mutagenesis studies have discovered several critical structural differences between FKBP51 and FKBP52, which are related to their function. The FKBP52-K354A mutation abolishes FKBP52 binding to Hsp90 and FKBP52 potentiation of receptor activity. Additional truncation mutants were generated to investigate whether the TPR domain is sufficient for potentiation of GR activity. Studies showed that all truncated FKBP52 mutants lacked the ability to potentiate receptor activity, which indicates that the TPR domain is not sufficient for FKBP52 potentiation. However, Hsp90 remains a critical binding partner allowing FKBP52-receptor interaction (23). In domain swapping experiments chimera constructs were generated in which the FKBP52's FK1 domain was exchanged. FKBP52 substituted with FKBP51 FK1 domain greatly decreased FKBP52-mediated enhancement of receptor activity. Interestingly, the converse substitution on FKBP51 caused a gain in potentiation, however it was only 50% the activity seen with FKBP52. (23) This suggests that the FK1 domain is important for FKBP52 function, yet other unidentified domains/regions still remain. Mutagenesis studies demonstrated that the FK2 domain may contain an interaction site that may confer FKBP51 and FKBP52 ability to identify a specific interaction partner, such as steroid hormone receptors. A gain-of-function random mutagenesis of FKBP51 generated an FKBP51 mutant that could potentiate receptor activity. The characterization of these mutants uncovered a proline-rich loop region that overhangs the PPIase catalytic pocket in the FK1 domain, which is critical for FKBP52's function. This loop contains several critical prolines at position 119 and 124 in FKBP52; the corresponding positions in FKBP51 are leucine and serine. Although the FKBP52

proline-rich loop is critical, mutations that make the FKBP52 loop more like that of FKBP51 only reduced receptor activity approximately 40%. Therefore, researchers concluded that there are additional unidentified regions or residues in FKBP52 that are essential for full function (16).

### 1.4 CURRENT RESEARCH FOCUS

Our lab is continuing to develop inhibitors specific to FKBP52, which is thought to bind the BF-3 region of the AR, thus augmenting its activity (110). Studies are currently being conducted to design inhibitors that specifically target FKBP52 regulation of the receptor through further characterization of its functional domains. A compound, MJC13, is predicted to bind to the BF3 surface (FKBP52 interaction site) that impairs the dissociation of the AR-Hsp90-hFKBP52 complex, resulting in an inhibition of AR nuclear translocation and AR dependent gene expression (110). As it stands current research has been limited in their capacity to specifically target AR: h52 interaction. In pursuit of this novel mechanism for targeting AR mediated gene expression further investigation into h52 structural and functional domains and residues is of vital importance.

Comparative analysis is a very powerful tool in research science. A select gene is compared with orthologous gene, allowing researcher to identify structural and functional domains, which was previously done using FKBP51. This study will be using an evolutionarily similar protein, *Danio rerio* FKBP52 (DrFKBP52), for a comparative analysis to help in understanding the relevant sequence differences that distinguish their ability to potentiate steroid hormone receptors. *Danio rerio* has been used in the past as an important vertebrate model organism in scientific research, the protein

DrFKBP52 activity within the steroid hormone complex is undocumented in current literature. Like h52, DrFKBP52 contains the 3 distinct domains: FK1, FK2, and a TPR domain. DrFKBP52 FK1 domain is comprised of the 9 amino acids that comprise the proline-rich loop. Its FK linker region, that connect the FK1 and FK2 domains contains a consensus casein kinase II (CKII) phosphorylation site, box outline (111). The FK2 domain is 85% conserved between DrFKBP52 and hFKBP52, and it also lacks an active PPIase domain. Both also contain the motif that binds HSP90 (2003). Despite their similarity at the amino acid level and structural level, DrFKBP52 and hFKBP52 are functionally divergent. Our thorough assessment of DrFKBP52 has led us to the conclusion that it would be an excellent model protein for gain-of-function comparative analysis with hFKBP52.

### 1.5 DISSERTATION GOAL

Because FKBP52 has an integral role in androgen receptor regulation and male sexual development, our lab is investigating it as a possible therapeutic target to treat Androgen-dependent and -independent diseases such as PC. Although previous research has identified several critical residues that are important for FKBP52 function, **we hypothesize that the FKBP52 FK1 and FK2 domains contain additional regions and/or residues required for function within the steroid hormone receptor complex.** While *Danio rerio* (zebrafish) FKBP52 (Dr52) possesses all known residues, regions, and domains known to be critical for FKBP52 function, Dr52 lacks the ability to potentiate receptor activity. We aim to further characterize functional regions through cross-species comparison with DrFKBP52 and human FKBP52 (h52), and to perform a gain-of-function random mutagenesis screen of Dr52 (6,40). Cross-species comparison

between two similar proteins with distinct functions has been used as a powerful tool in research. This approach will allow us to further map out and characterize functional regions important for FKBP52's full function. The overall goal of this work is to further delineate functional regions in hFKBP52 that are critical for its function and characterize AR-h52 interaction sites, which will ultimately serve as therapeutic drug targets

# CHAPTER 2: FUNCTIONAL AND STRUCTURAL COMPARISON OF HUMAN AND DANIO *RERIO* FKBP52 REVEALS A NOVEL DOMAIN IMPORTANT FOR hFKBP52 FUNCTION

## 2.1 RATIONALE

Current literature has established FKBP52 as a critical player in the development and integrity of the male reproductive system and male phenotype (3). This, along with other recent developments, implicates FKBP52 as a relevant factor in PC, AIS, and endometriosis, which makes FKBP52 an attractive therapeutic target. Biochemical and cellular studies demonstrated that FKBP52 potentiates steroid hormone receptor signaling pathways. As we seek to target AR-FKBP52 interactions as a novel approach to PC therapy, determining functional domains (i.e. potential drug binding sites) has become increasingly important in developing FKBP52 specific inhibitors. Prior to this study, Riggs et. al (6). identified a proline-rich loop region, which overhangs the catalytic PPIase pocket, that is thought to be important for FKBP52 functional activity and interaction with AR. This loop was identified through a random mutagenesis screen of FKBP51; a protein that is 70% similar to FKBP52, but that does not potentiate receptor function. Although this region is critical for FKBP52 function, further investigation indicates that other residues may also contribute to FKBP52 function. This idea is reinforced by the fact that mutations which make the FKBP52 proline-rich loop more like that of FKBP51 only reduce FKBP52 activity by approximately 40%. Thus, ***our initial hypothesis was that additional important residues remain to be identified in the FKBP52 FK1 and/or FK2 domains.*** To investigate this hypothesis, we performed a cross-species comparison with other FKBP proteins, created chimera constructs using *DrFKBP52* and hFKBP52, evaluated the chimeric proteins' function in yeast-based assays, assessed the chimeric proteins for the ability to associate with Hsp90, and corroborated our results in a higher vertebrate

model system (52KO mouse embryonic fibroblasts). This approach allowed the further mapping and characterization of functional regions that are critical for FKBP52 function within the SHRC.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Construction of FKBP52 And DrFKBP52 Chimeras

For the HZ-96 and ZH-262 chimeras, chimeric cDNAs combining hFKBP52 and DrFKBP52 cDNA sequences were constructed by PCR. Primers for the two fragments were designed to contain the complementary sequences that surround the desired fusion site. The resulting DNA products are gel-purified and used as megaprimers in a reaction with the appropriate 5' - 3' primers to generate the full-length chimeric cDNA. The final PCR product was ligated into pSPUTK (Stratagene) for *in vitro* expression, or into a yeast expression vector.

For the HZ-139, HZ-258, and ZHZ-150_250 chimeras, chimeric cDNAs combining hFKBP52 and DrFKBP52 cDNA sequences were constructed by yeast recombination method. Yeast has the unique ability to recombine any template that has homologous overlapping regions when co-transformed with another template or linearized vector containing the homologous regions. Primers for the appropriate fragments were designed to contain the complementary sequences that surround the desired fusion site. Fragment templates were PCR amplified with desired overlapping regions homologous to the fusion site, the PCR product was gel purified and quantified. Double or single digestions were performed on *DrFKBP52 and hFKBP52* in the desired cloning region, the reactions were gel purified and quantified. The amplified fragment

and linearized plasmid were co-transformed into yeast (W303a) in a 1:10 ratio. Transformed yeast were streaked on selective media plates and incubated at 30°C for 4 days. Several colonies were picked from the plate and cultured overnight in appropriate selective media. Plasmids were then extracted from yeast using standard procedures and sequenced to verify correct construction of the chimeras.

### 2.2.2 *In Vitro* Protein Binding Assays

Radiolabeled chimeric proteins were generated by *in vitro* transcription/translation (TNT kit, Promega, Madison, WI) in the presence of [$^{35}$S]-methionine. A 5 µl aliquot of each synthesis mixture was separated by SDS- PAGE, followed by autoradiography. Protein bands were quantified by densitometry. For each Co-immunoprecipitation, molar equivalents of each radiolabeled protein was added to 100 µl of rabbit reticulocyte lysate (RL) (1:1 lysate; Green Hectares, Oregon, WI) supplemented with an ATP-regenerating system. The RL mixture was added to the immunoresin (a 10 µl pellet pre-adsorbed with 40 µg of Hsp90 antibody) and incubated for 90 min at 30°C. Washed, resin-bound complexes were separated by SDS-PAGE, Coomassie-stained, and autoradiographed.

### 2.2.3 Yeast Assays for Hormone-Induced Reporter Gene Expression

β-galactosidase reporter assays were used as a quantitative indicator of steroid hormone receptor activity. Parental strains were co-transformed with three plasmids: a constitutive receptor expression plasmid (p415GPD-GR, p425GPD-hAR, or p425GPD-hAR-P723S), a hormone-inducible β-galactosidase reporter plasmid (pUCΔS-26x), and a plasmid constitutively expressing a chimera variant. Isolates were cultured at 30°C overnight (12-16 hours) in 5 mls of selective media (SC-LUW). Overnight cultures were

diluted back to an optical density at 600 nm ($OD_{600}$) of 0.08,and growth was monitored by spectrophotometry for 30-60 min before hormone addition to ensure that the cultures were in exponential phase growth. Deoxycorticosterone (DOC) or Dihydrotestosterone (DHT) were added to the culture at 0, 100, and 500 nM and 0, 3, 5, and 10 nM final concentrations respectively. To assay for galactosidase activity 100 µl of culture was withdrawn and immediately added to 100 µl of the Gal-ScreenTM substrate (Applied Biosystems, Bedford, MA) in 96-well microtiter plates at room temperature. One sample is taken from each assayed isolate 120 min after hormone addition and read in a luminometer. To determine the degree of reporter expression, galactosidase induction curves were first generated by plotting relative light units (RLU) against the absorbance at 600 nm of the culture. The normalized levels of reporter expression are reported ($OD_{600}$/RLU) and are all normalized to 100% expression. Data is represented as the mean (+/- standard deviation) of at least three replicate samples.

**2.2.4 Western Blot of Yeast Lysates**

Yeast cells in exponential phase growth ($OD_{600}$ approximately equal to 1) were pelleted, washed in 1ml of lysis buffer, and then resuspended in 5 mls lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol supplemented with protease inhibitors), and vortexed vigorously in the presence of glass beads for 8 x 2 min. Cell extracts were clarified at 14,000 rpm for 20 min at 4°C. Protein concentrations in yeast extracts were determined using Coomassie Plus (Pierce, Rockford, IL). Typically, 20 µg of total cellular protein per lane was separated on a 10-20% Criterion gel (BioRad, Hercules, CA) and transferred to PVDF membranes. Mouse monoclonal antibodies Hi52D, Hi52B were used to detect hFKBP52 and DrFKBP52, respectively. As protein loading

controls, yeast extracts were blotted with antibody recognizing the L3 ribosomal subunit (housekeeping gene). The secondary antibody was an alkaline phosphatase-conjugated goat anti-mouse antibody (Pierce, Rockford, IL), and bands were visualized with Immun-Star AP Substrate (BioRad, Hercules, CA) and expose to X-Ray film.

## 2.3 RESULTS

### 2.3.1 Danio rerio FKBP52: A Comparative Model for Human FKBP52 Characterization

Our current knowledge of the characteristics that contribute to FKBP52 potentiation of receptor activity was thought to be predominately governed by the proline-rich loop region that overhangs the PPIase catalytic pocket. Previous studies corroborate the importance of this domain in a gain-of-potentiation random mutagenesis screen performed on FKBP51 (6). However, chimeric studies with FKBP51 and FKBP52 suggest that additional residues may exist within hFKBP52, particularly in the FK2 domain, that are critical for function. Thus, we decided to do a cross-species comparison of hFKBP52 with ten other species of FKBP52 and hFKBP51, using clustalW alignment software, focusing close attention on the proline-rich loop region (Table 2.1). Of these species, we observed that DrFKBP52 is approximately 61% similar in amino acid sequence to hFKBP52. Upon further investigation it was discovered that DrFKBP52 contains all of the regions and residues identified by previous studies as critical for hFKBP52 function: proline rich loop and residues critical for binding Hsp90 (Figure 2.1). Although *Danio rerio* has been used in the past as an important vertebrate model organism in scientific research, DrFKBP52 function

within the steroid hormone receptor heterocomplex is undocumented in the current literature. Like hFKBP52, DrFKBP52 contains the three distinct domains, FK1, FK2, and TPR domains, highlighted in grey in figures 2.1 and 2.2. The DrFKBP52 FK1 domain contains the 6 amino acids that comprise the proline-rich loop.

## Table 2.1: ClustalW2 Alignment of FKBP Species

| FKBP52 | Gene bank# | P-Loop | CKII | HSP90 (YANMF) | %Homology | #Amino Acids | Functional |
|---|---|---|---|---|---|---|---|
| *Macaca mulatta* | NP_001248268 | Y | Y | Y | 93.3% | 459 | Y |
| *Mus musculus* | AAH03447.1 | Y | Y | Y | 89.96% | 612 | Y |
| *Oryctolagus cuniculus* | NP_001075779 | Y | Y | Y | 91.27% | 458 | Y |
| *Rattus norvegicus* | EDM01775.1 | Y | Y | Y | 90.17% | 560 | Y |
| *Mus musculus* | AAH03447.1 | Y | Y | Y | 89.96% | 612 | Y |
| *Equus caballus* | XP_005610895 | Y | Y | Y | 87.58% | 414 | UNKNOWN |
| *Monodelphis domestica* | XP_001366229 | Y | Y | Y | 81.05% | 462 | UNKNOWN |
| *Gallus gallus* | NP_001006250.1 | Y | Y | Y | 74.66% | 442 | Y |
| *Danio rerio* | AAI65584.1 | Y | Y | Y | 61.02% | 449 | N |
| *hFKBP51* | AAH42605.1 | N | N | Y | 54.7% | 448 | N |
| *Echinococcus granulosus* | CDJ23395 | N | N | N | 29.86% | 432 | UNKNOWN |

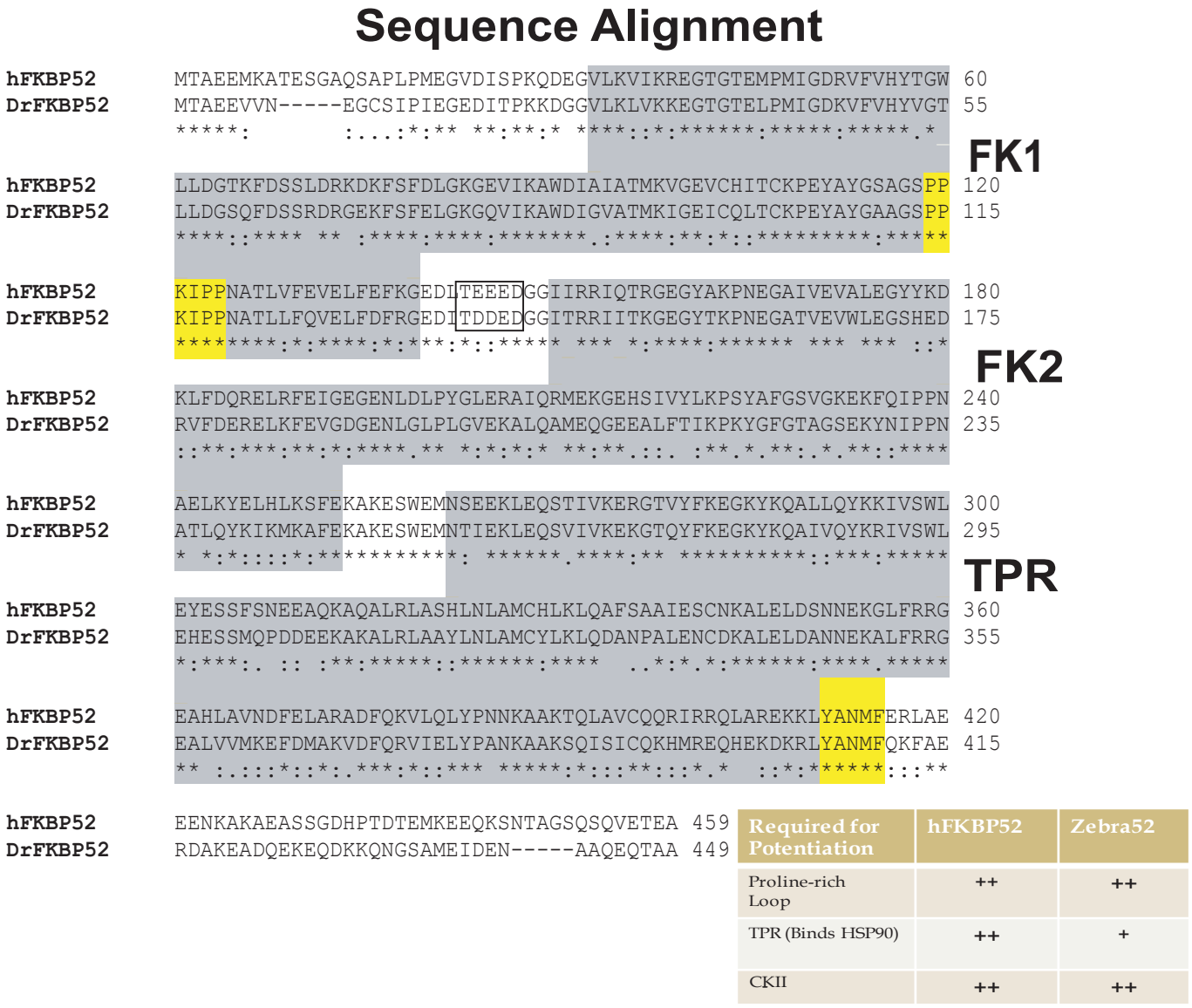



**Figure 2.1: Sequence Alignment of hFKBP52 and DrFKBP52.**

Figure 2.1: Sequence Alignment of hFKBP52 and DrFKBP52.

ClustalW2 alignment tool was used to align hFKBP52 and DrFKBP52. Dr52 is ~61% homology to hFKBP52. Identified in gray are the 3 major domains: FK1, FK2, and TPR domains. Highlighted in yellow are the previously characterized critical domains: Proline-rich loop and conserved residue indicative of Hsp90 binding. Boxed in black is the CKII phosphorylation site. In the right corner is a table indicating comparative activity relative to domains.

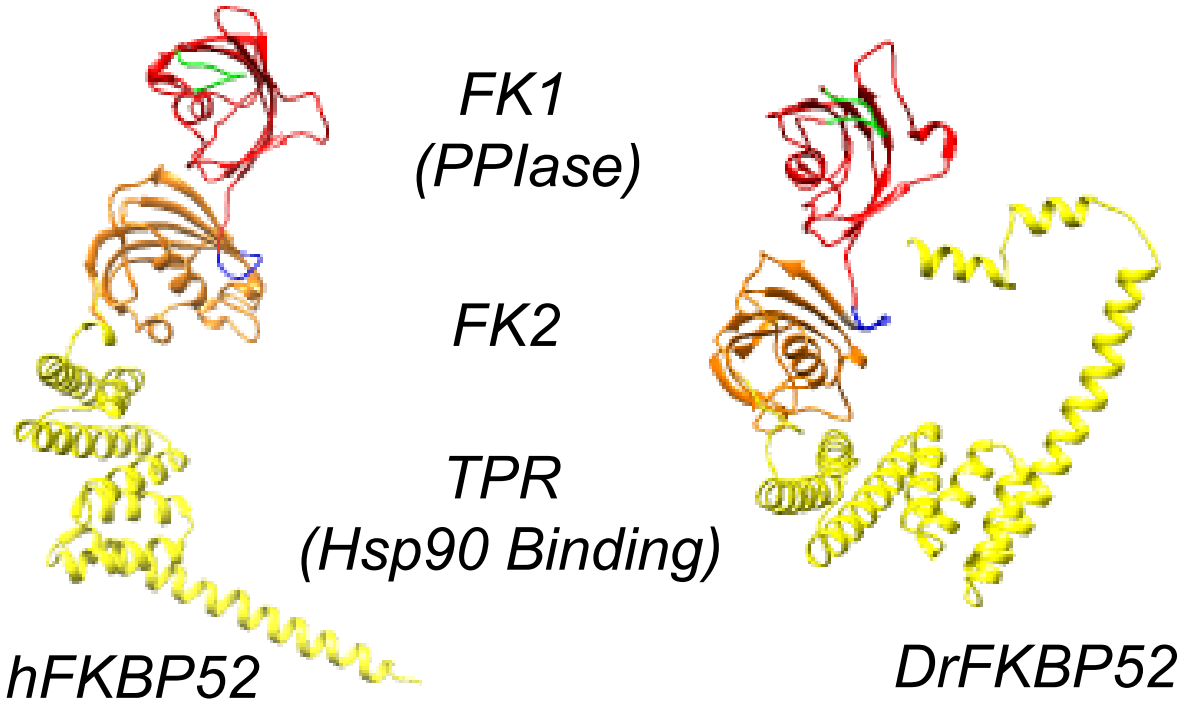



**Figure 2.2: 3D Crystal Structure of hFKBP52 and DrFKBP52.**

The three-dimensional structure of the composite of two partial structures for human FKBP52 (protein databank: 1Q1C and 1P5Q) and the structure of human DrFKBP52 (Homology modeling) are shown in ribbon format. The important functional regions FK1 (red), FK2 (orange), TPR (yellow), Blue is the FK1 linker and the proline loop (Green), are illustrated. The figure was created using UCSF Chimera version 1.5.

The FK linker region that connects the FK1 and FK2 domains contains a consensus casein kinase II (CKII) phosphorylation site (TEEED, TDDED) (108, 111) The FK2 domain is 85% conserved between DrFKBP52 and hFKBP52, and lacks an active PPIase active site. Both contain a conserved motif, YANMF, that binds Hsp90 (24). In addition, we have superimposed the FK1 and FK2 domains of DrFKBP52 and hFKBP52 and they have a high degree of similarity, and adopt a similar conformational fold (Figure 2.3). Despite their similarity at the amino acid and structural level, DrFKBP52 and hFKBP52 are functionally divergent (Figure 2.4). A functional yeast assay reveals that DrFKBP52 does not confer the ability to potentiate human AR or GR, like hFKBP52 as receptor activity in the presence of a plasmid expressing DrFKBP52 is comparable to that in the presence of the empty vector control (Figure 2.3). Riggs et. al. identified the highly conserved proline-rich loop region as being critical for hFKBP52 activity (6), and, although DrFKBP52 has a similar proline-rich loop, it does not have the ability to augment receptor activity. Hsp90 binding studies show that DrFKBP52 has the ability to bind Hsp90, yet at a decreased affinity (Figure 2.5). It must be noted that preferred binding of hsp90 is not an accurate indicator of a protein's ability to potentiate receptor activity. This is seen in the preferential binding of FKBP51 to hsp90, yet FKBP52 has a reduced affinity for Hsp90 (24). Based on these data, DrFKBP52 is a highly similar protein to hFKBP52, yet lacks function. Thus, DrFKBP52 can serve as an excellent model for comparative analysis with hFKBP52.

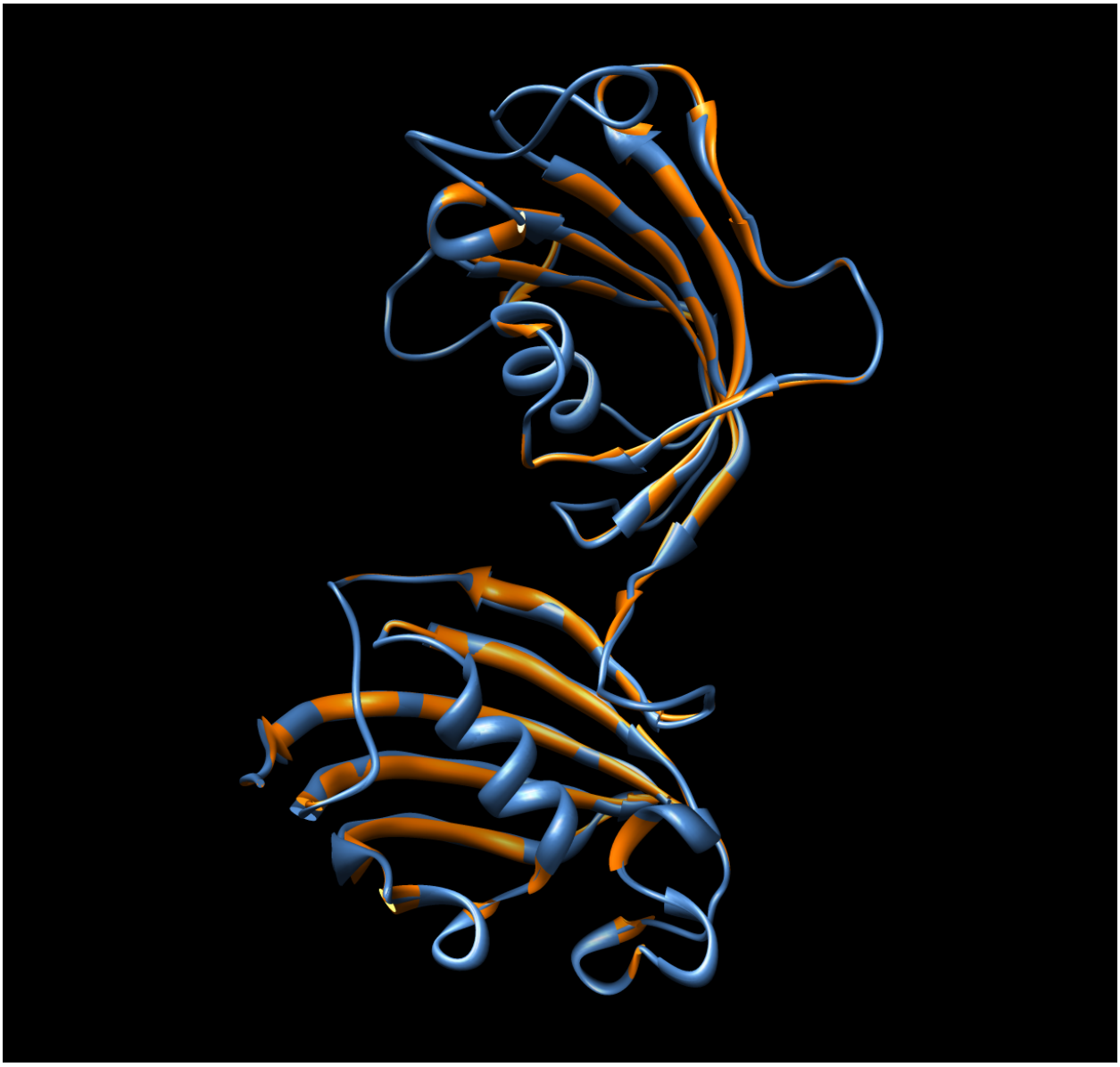


**Figure 2.3: Danio rerio and Human FKBP52 FK1 and FK2 Domains Superimposed.** The DrFKBP52 (orange) and hFKBP52 (blue) 3D crystal structure were superimposed to identify structural similarities and differences.

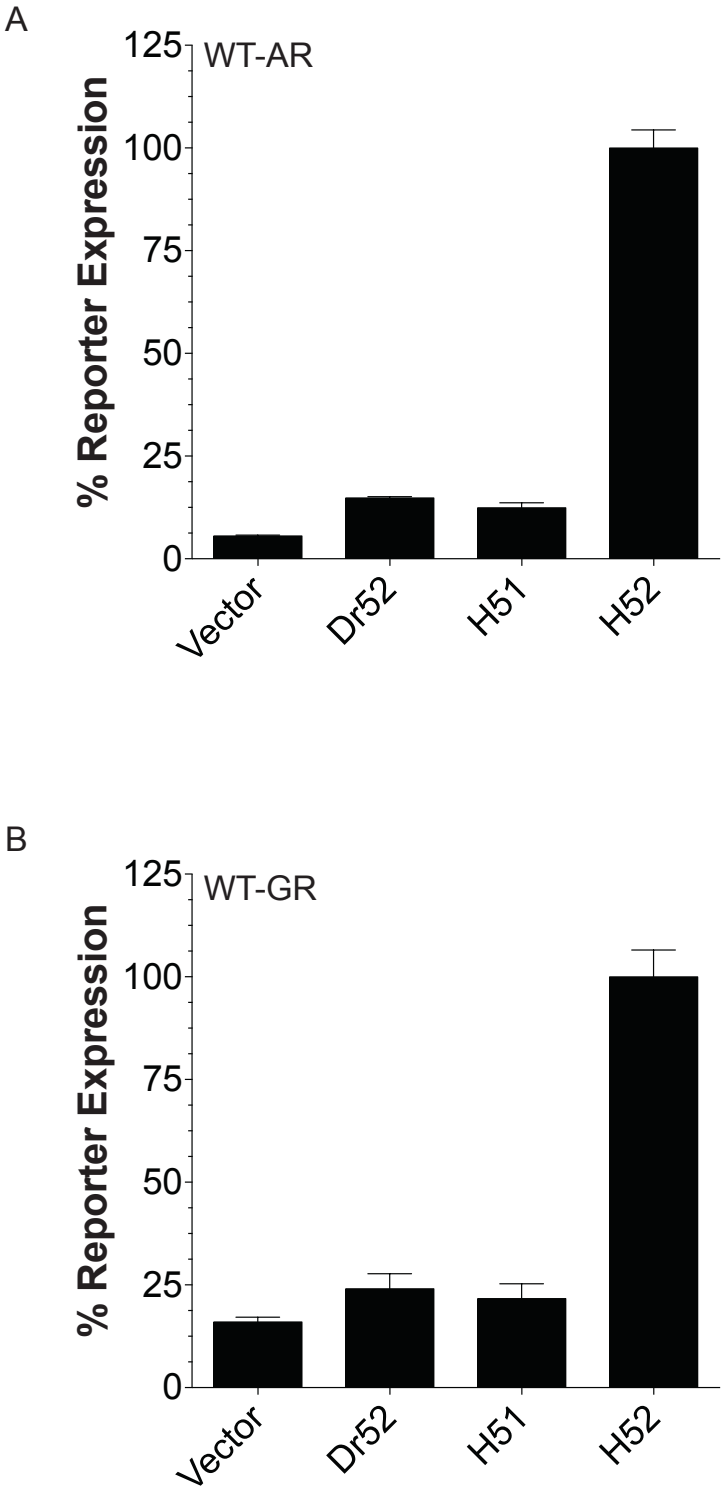



**Figure 2.4: Functional AR and GR Yeast Assay of Danio rerio FKBP52**

Figure 2.4: Functional AR and GR Yeast Assay of *Danio rerio* FKBP52

Vector, FKBP52, FKBP51 and Dr52 were co-transformed into wt-AR or GR. Cells were incubated for 2 hours with 3nM DHT or 500nM DOC. Three isolates were picked form each strain and was replicated at least 3 times. AR and GR expression was measured by evaluating reporter expression/relative light units (RLU), followed by a normalizing with the final $OD_{600}$ reading of cultured isolates. Bars represent the average reporter expression of a least 3 replicates (RLU/$OD_{600}$ with SD).

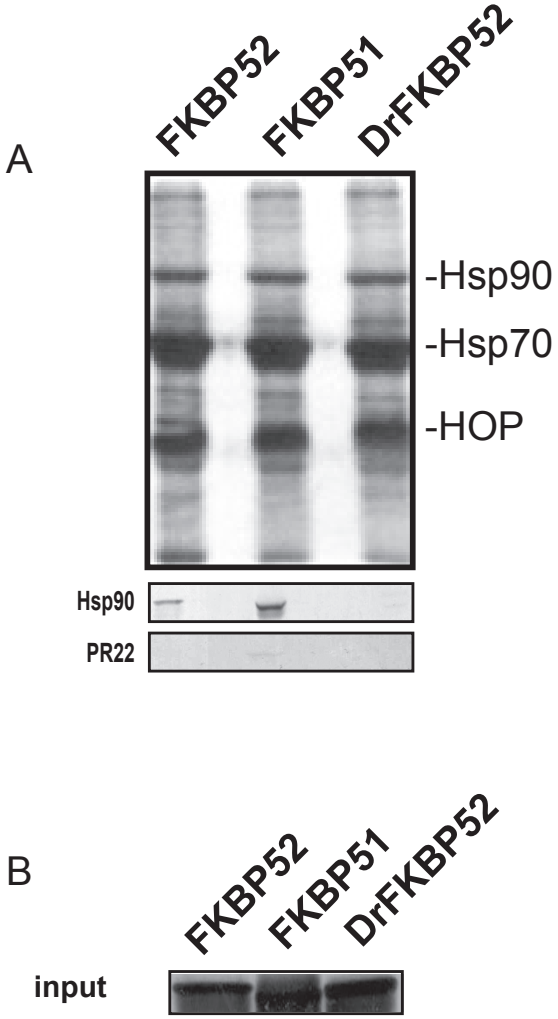



**Figure 2.5: Hsp90 Binding FKBPs**

(A) Co-IP of hFKBP52, hFKBP51, DrFKBP52 with HSP90. Pr22 is anti-PR and is used as a non-specific binding control. Hsp90 was immunoprecipitated with radiolabeled ($^{35}$S-methionine) hFKBP52, hFKBP51 and DrFKBP52. Proteins were separated by SDS-PAGE, coomassie-stained, and autoradiographed (B) Input: loading control.

### 2.3.2 Functional Assessment Of Chimera Constructs In Yeast And Mammalian Cells

To further characterize the role of individual FKBP52 domains in the functional maturation of steroid hormone receptors we created hFKBP52 and *Dr*FKBP52 chimeras by interchanging the FK1, FK2 and TPR domains. We generated five hFKBP52/DrFKBP52 chimeras and evaluated their functional relevance in yeast. These chimeras were designed and generated through various experimental techniques: mega primer PCR, PCR, Yeast recombination and fusion cloning. Each chimera was meticulously constructed and sequence analysis was performed to verify the correct sequence. The chimeras were transformed into three different yeast strains that express a variety of receptors and their respective β-galactosidase reporter plasmids including wild type AR (Figure 2.6B), GR (Figure 2.6C), or AR P723S (Figure 2.6E). The ability of the chimeric proteins to potentiate receptor function was assessed by hormone-induced reporter assays.

We first evaluated the relevance of the hFKBP52 TPR domain, Chimera HZ-262, in the regulation of the steroid hormone receptors. Though TPR domains are highly conserved across species, there is a possibility that it plays a role in structural integrity, or has a specific interaction with the FK1 or FK2 domain given that the DrFKBP52 tail is notably more kinked than that of hFKBP52.

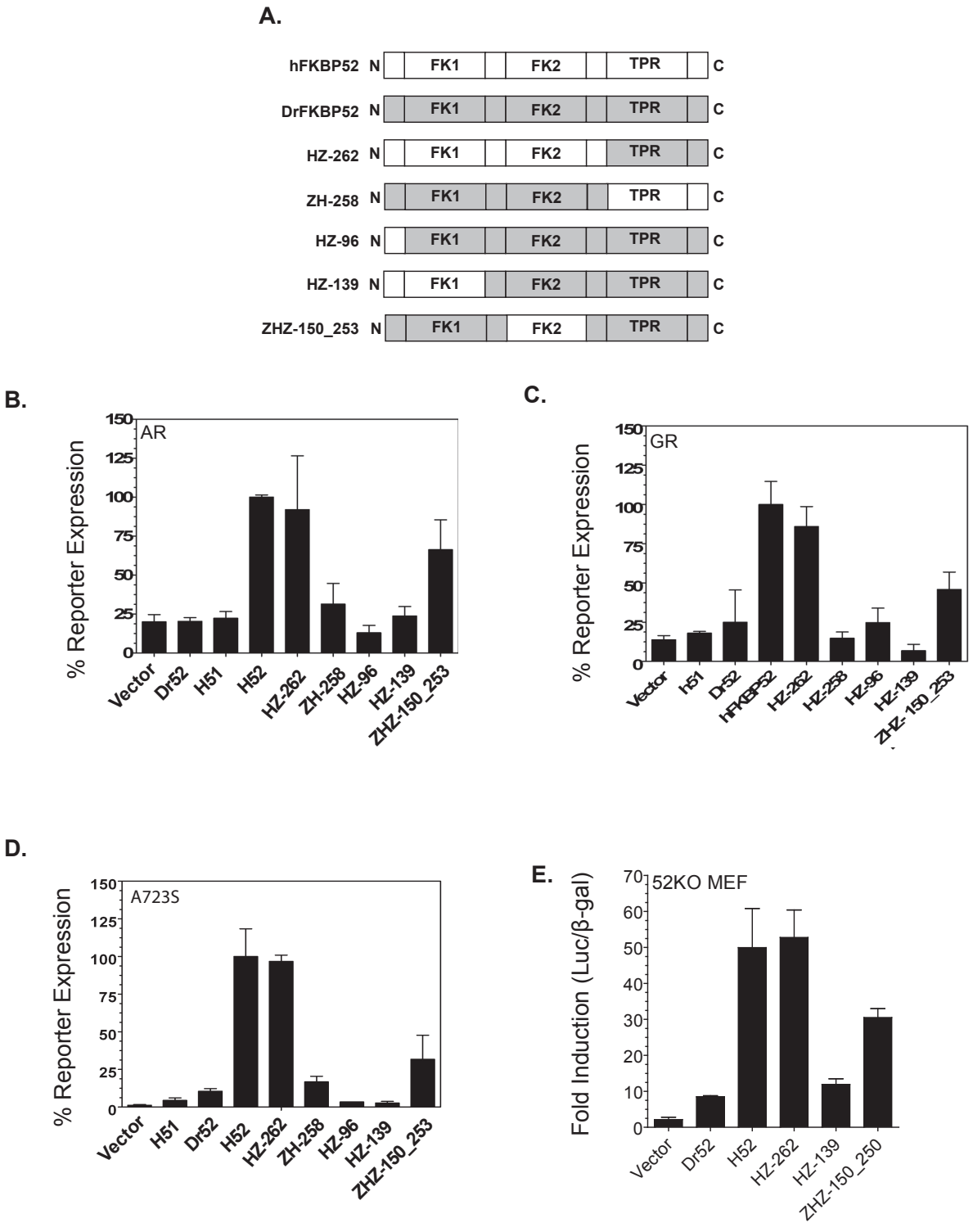



**Figure 2.6: Functional Analysis of FKBP Chimeras.**

Figure 2.6: Functional Analysis of FKBP Chimeras.

(A) As diagrammed at the top of the figure, chimeric proteins were generated by exchanging FK1, FK2 and TPR domains between DrFKBP52 (Dr52) (shaded) and hFKBP52 (52) (white). The activities and expression levels of the FKBP constructs were assayed in yeast containing (B) wt-AR, (C) AR:P723S or (D) GR. (E) Chimera constructs activity was also assessed in 52KO MEF containing wild-type AR and an appropriate hormone-dependent reporter. Chimeras were detected in cell extracts by Western immunostaining with monoclonal antibodies specific for either the FKBP52 FK1 domain (Hi52B). Bars represent the average reporter expression of a least 3 replicates (RLU/$OD_{600}$ with SD). Using the one way Anova statistical analysis following a post turkey test with an alpha value of 0.05, AR, GR, AR:P7233 reporter activity is significantly enhanced in cells transfected with Chimera ZHZ-150_250 and Chimera HZ-262 as compared to those transformed with either Dr52 and HZ-139 when comparing cells enhanced with DHT, with a p-value $< 0.01$. AR reporter activity is also significantly enhanced in 52KO MEF cells transfected with Chimera ZHZ-150_250 as compared to those transfected with either Dr52 and HZ-139 when comparing cells enhanced with DHT, with a p- value $< 0.001$.

Chimeras containing the hFKBP52 FK1/FK2 and DrFKBP52 TPR domains retains the greatest ability to potentiate all receptors, demonstrating that the TPR domain is conserved and functionally interchangeable. This fact was further established given that Chimera ZH-258 (DrFKBP52:FK1/FK2 and hFKBP52:TPR) activity is comparable to vector (Figure 2.6). Because the hFKBP52 FK1 domain has been identified as the main contributor to receptor activity we assessed its function in combination with DrFKBP52. FK1 has also been predicted to be the domain that contacts the BF3 surface on AR. Surprisingly, Chimera HZ-139 (hFKBP52:FK1 and DrFKBP52: FK2/TPR) was unable to potentiate receptor activity like hFKBP52. Because hFKBP52's FK2 domain is a poorly understood domain and has been previously identified as a non-functional domain, we sought to determine its significance in hFKBP52 activity. DrFKBP52 and hFKBP52 are approximately 85% similar in amino acid sequence identity. However, Chimera ZHZ-150_253 (DrFKBP52: FK1/TPR and hFKBP52:FK2) has a statistically significant increase in activity in AR and GR, as compared to DrFKBP52. In comparison with HZ-139 that retains the FK1 domain, chimera ZHZ-150_253 has a marked improvement in receptor potentiation. The FK2 domain has thus far been characterized as a redundant domain with no identifiable significance, but these data identified a potential role for the hFKBP52 FK2 domain. A functional yeast assay using AR-P723S was also done to further solidify chimera functional relevance, as AR- P723S is hyperdependent on hFKBP52 for activity. Results substantiate chimera HZ-262 role in restoring receptor function like hFKBP52. Chimera ZHZ-150_253 restores partial receptor activity, while chimera HZ-139 could not. Yeast data was corroborated in 52KO mouse embryonic fibroblast cells, which further corroborates the previous data. Collectively this data gives

us new insight into the role of the FKBP52 FK2 domain in receptor modulation and could be a possible target for FKBP52 specific inhibitors (Figure 2.6).

### 2.3.3 Characterization of the FK2 Domain

Our pervious data clearly indicate hFKBP52 FK2 domain as a regulator of steroid hormone receptor activity. This discovery has led us to evaluate whether specific residues can be identified in the FK2 domain that specifically contribute to hFKBP52's ability to modulate receptor function. In an effort to isolate critical residues in the FK2 domain we have done a comparative analysis of DrFKBP52, hFKBP52, and hFKBP51. It has been determined that the divergent factor between hFKBP52 and hKBP51 is the proline-rich loop region, suggesting that the important residues in the FK2 domain are likely the same in hFKBP51 and hFKBP52. We have utilized this fact in identifying residues that differ in DrFKBP52 as compared to both hFKBP52 and hFKBP51. We mutated residues in DrFKBP52 in hopes that it would restore its ability to augment receptor activity. Using this comparative approach we identified 8 residues that are not conserved between DrFKBP52 and hFKBP52 (Figure 2.6). This rational approach allowed us to identifying possible important residues. Residues were identified and site-directed mutagenesis was preformed to mutate residues on DrFKBP52. These mutants were co-transformed with wild type AR and yeast assays were preformed to determine functional significance. Results indicated that none of the mutants except I145T was sufficient in potentiating receptor activity. DrFKBP52-T145I shows an approximately 50% increase in receptor activity as compared to wild type DrFKBP52 (Figure 2.7).

A.

IDENTIFICATION OF NON-CONSERVED RESIDUES IN FK2 DOMAIN

```
hFKBP52    150 IRRIQTRGEGYAKPNEGAIVEVALEGYYKDKLFDQRELRFEIGEGENLDLPYGLERAI 208
DrFKBP52   144 TRRIITKGEGYTKPNEGATVEVWLEGSHEDRVFDERELKFEVGDGENLGLPLGVEKAL 203
hFKBP51    147 IRRTKRKGEGYSNPNEGATVEIHLEGRCGGRMFDCRDVAFTVGEGEDHDIPIGIDKAL 206
                **   :***: .**:** **: :** .: ** *:: * :*:*:. .:* *::.*: :

hFKBP52    209 QRMEKGEHSIVYLKPSYAFGSVGKEKFQIPPNAELKYELHLKSFE 253
DrFKBP52   204 QAMEQGEEALFTIKPKYGFGTAGSEKYNIPPNATLQYKIKMKAFE 246
hFKBP51    207 EKMQREEQCILYLGPRYGFGEAGKPKFGIEPNAELIYEVTLKSFE 248
               *:: *..:. : * *.**  *. :: * *:*** * *:: :*:**
```

B.

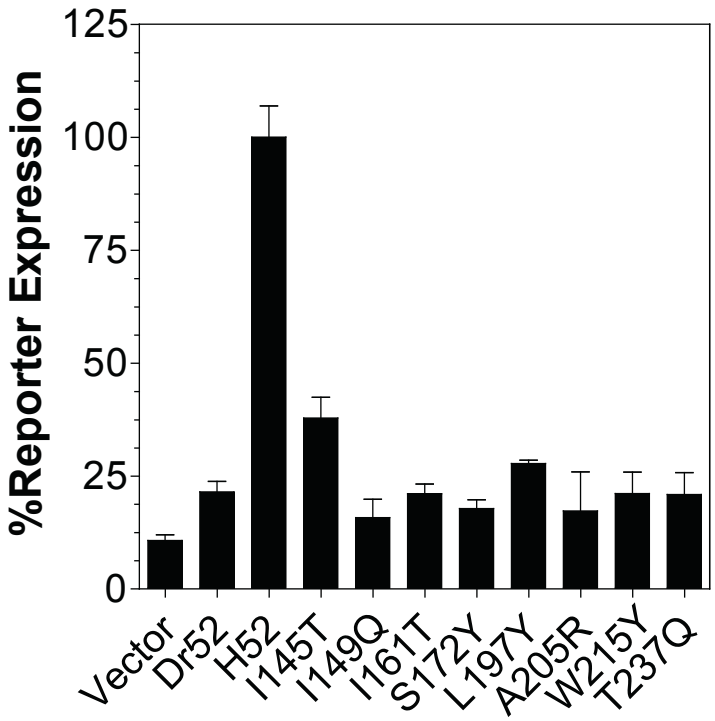



**Figure 2.7: FK2 Domain Comparison.**

Figure 2.7: FK2 Domain Comparison.

(A) As diagrammed at the top of the figure, the FK2 domain of DrFKBK52, hFKBP51 and FKBP52 were aligned and non-conserved residues where identified and Site-directed mutagenesis preformed. Mutated residues are highlighted in yellow. The activities of the FKBP constructs were assayed in yeast containing (B) wt-AR. Bars represent the average reporter expression of a least 3 replicates (RLU/$OD_{600}$ with SD).

However, no functional significant was seen when co-transformed with AR-P723S. Though this study was not successful in allowing us to identify specific domains within the FK2 domain that significantly restored activity, it does suggest a possible interest in the FK linker and residues in the surrounding area. Structural studies have identified this region as palpable and flexible, allowing for various interactions with the FK1 domain (112).

## 2.4 DISCUSSION

The generation of cross-species chimera constructs allowed for the investigation of the domain-specific importance of the hFKBP52 protein. Chimera constructs have been utilized in the past, using the closely related and structurally similar hFKBP51. Those studies demonstrated that FKBP51 and FKBP52 functionally diverged by two residues within the FK1 proline-rich loop, and demonstrated that the proline-rich loop is critical for FKBP52 regulation of receptor function. Utilizing a similar comparative approach allowed our lab to take advantage of the unique characteristic found in DrFKBP52. DrFKBP52 is similar to hFKBP52, and contains all of the critical domains and residues known to be critical for receptor potentiation. The failure of DrFKBP52 to modulate receptor activity like hFKBP52 is evidence that additional important residues remain to be identified. Whether or not human FKBP52 directly interacts with the receptor is unknown, but its association with Hsp90 is thought to bring FKBP52 into close proximityto the receptor. However, the fact that FKBP52 displays receptor specificity suggests that there is a direct receptor:hFKBP52 interaction, facilitated through a specific surface contact. Identifying these residues, domains, or motifs are of critical importance for the aim of targeting hFKBP52 as a prospective PC therapeutic

agent.

Through this study our lab has come a step closer to establishing a functional role for the FK2 domain in FKBP52. Chimera studies involving hFKBP52 and DrFKBP52 strongly imply that the FK2-domain is an important regulator of FKBP52 activity. This study also suggests that the FK2 domain may be more important in augmenting AR:FKBP52 activity than the critical proline-rich loop. This is not surprising as recent studies by (112) identified a dynamic and fluid interaction between the FK1 and FK2 domain, facilitated by the FK1-linker region. Rather than simply being redundant and non-functional, the FK2 domain could very well be a key player in receptor activity as indicated by Chimera ZHZ-150_253.

In summary, we have revealed a unique role for the FKBP52 FK2 domain in potentiating AR and GR activity. The FK1 domain of hFKBP52, in exchange, did not potentiate receptor activity. This paradox is unexplainable, as past studies have shown that the FK1 domain is critical for activity. There could be a structural restriction and consequences when exchanging domains that are not identifiable by biochemical studies and would need further probing to determine. Be this as it may, the sole contributor to Chimera ZHZ-150_253 activity is the hFKBP52 FK2 domain, which places significant weight on its importance in hFKBP52's activity in regulation of steroid hormone receptors. The FK2 domain has not been evaluated as a possible target, but might be a plausible option now that we know it has an important role in hFKBP52 activity.

# CHAPTER 3: FUNCTIONAL RANDOM MUTAGENESIS OF hFKBP52 IN COMPARISON TO DANIO *RERIO* FKBP52

## 3.1 RATIONALE

As stated previously in section 2.1, the studies using FKBP51 identified residues important for hFKBP52 activity. However, critical residues remain to be identified. We hypothesize that other residues exist within the hFKBP52 FK1 and/or FK2 domains that are required for full activity. Random mutagenesis was utilized with great success to identify the divergent differences in FKBP51 and FKBP52, thus we will utilizes this same approach utilizing differences between DrFBKP52 and hFKBP52.

## 3.2 EXPERIMENTAL DESIGN

### 3.2.1 Yeast Strains and B-Galactosidase Reporter Assays

The β-galactosidase reporter assays that were used as a quantitative measure of receptor activity were described previously (25). For hormone-responsive reporter assays, the indicated strains were co-transformed with three to four plasmids: a hormone-inducible β-galactosidase reporter plasmid (pUCΔss-26X [reporter]), a plasmid constitutively expressing the indicated steroid hormone receptor from a glyceraldehyde phosphate dehydrogenase (GPD) promoter, and high-copy number plasmids expressing yeast parent vector, human FKBP52, Danio rerio FKBP52, and/or site-directed mutants where indicated. Human AR and the mutant AR-P723S were cloned, respectively, into p424GPD and p424TEF. All hormones were obtained from Sigma (St. Louis, MO) and were stored as 50 mM stock solutions in ethanol. Hormone dilutions were setup so that the ethanol vehicle never exceeded 1% in the yeast cultures. The hormone concentrations were optimized in order to maximize the difference between cells carrying an empty vector versus cells carrying an human FKBP52 expression

vector by performing dose-response curves. Hormone-induced reporter activity was measured from yeast extracts as described previously with a single two-hour time point measurement. To determine the rate of reporter expression, galactosidase induction curves were first generated by plotting relative light units (RLU) against the A600 of the culture sample ($OD_{600}$/ β- galactosidase RLU). The normalized levels of reporter expression are normalized to 100% expression and reported in graphical form using GraphPad. All assays were performed at least in triplicate and the data shown are representative of at least three independent experiments that produced consistent results.

### 3.2.2 Site-Directed Mutant Plasmids

Mutations were introduced by site-directed mutagenesis (QuikChange II XL; Stratagene, San Diego, CA) into the wild-type human FKBP52 or Danio rerio FKBP52 gene cloned into p423GPD. Potentiation by these mutant FKBPs was measured in strain W303a transformed with the reporter plasmid (pUCΔss-26X) and AR or AR P723S expression plasmid. Dihydrotestosterone (DHT) (3 nM or 10 nM stock) was used in the wild-type AR and AR-P723S assays.

### 3.2.3 Selection for FKBP51 Potentiation Mutants and Analysis

The mutant FKBP51 library was made using error-prone PCR (GeneMorphII; Stratagene, CA) using the manufacturer's recommended conditions for Low-frequency mutagenesis (50-100ng target DNA per reaction). The template used was p425GPD-DrFKBP52, and the primer binding sites were approximately 100 bases outside of the gene borders. The PCR product (400 ng, purified by agarose gel electro- phoresis) and p424GPD vector (100 ng, linearized with EcoRI and SalI) were cotransformed into the

selection strain YNK435 (*MAT***a** *ura3-52 lys2-801 ade2- 101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 pdr5*::*GT3Z his3*::*GT3H*) containing the plasmid p425TEF-AR-P723S. DrFKBP52 potentiation mutants were selected on plates containing synthetic complete medium lacking tryptophan, leucine, and histidine (SC-WLH) supplemented with 10 nM DHT and 10 mM 3-amino-1,2,4-triazole (3AT). The concentrations of each were adjusted to maximize the growth differential between YNK435 strains expressing either hFKBP52 or DrFKBP52. The colonies that grow comparable to hFKBP52 were selected. The mutant phenotype (potentiation of hormone signaling) was confirmed by assaying hormone-dependent expression of the β-galactosidase reporter in the selection strain. Isolates that showed increases in both hormone-dependent growth and β-galactosidase expression were confirmed by transferring the mutated DrFKBP52 gene into a clean genetic background. This was done by extracting the mutated DrFKBP52 from yeast lysates. The extracted mutant was transformed into strain W303a expressing AR-P723S and containing a plasmid-encoded hormone-responsive β-galactosidase gene (pUCΔss-26X). Transformants were assayed for potentiation of hormone signaling as described above. Those mutated DrFKBP52 genes that retained the potentiation properties in this clean genetic background were then sequenced, and interesting mutations were put in individually and in combination into the DrFKBP52 gene by site-directed mutagenesis.

### 3.2.4 Yeast Plasmid Extraction

Cells are selected and cultured overnight in selective media at 30°C. Cells were then lysed according to a standard Western blot protocol. Cell lysates were then minipreped (Pure Yield Plasmid miniprep system, Promega Madison, WI). Samples

were eluted off of the column with 50 µl of cold nucleus free water. Samples were than transformed into DH5α and plated on Luria Bertani media with Ampicillin.

### 3.2.5 Hormone-Induced Gene Expression In Mouse Cells

For assays, immortalized mouse embryonic fibroblasts (MEF) derived from FKBP52 knockout mice (Tranguch, S., et al 2005), were cultured in 5% $CO_2$ in HyClone Minimal Essential Media/Eagles Essential Salt Solution with 2 mM L-glutamine (Thermal Scientific, Logan, UT) supplemented with 10% Charcoal/Dextran Treated Fetal Bovine Serum (FBS) (HyClone, Logan, UT) 24 hours prior to the transfection. Cells were cultured in 6-well plates until they were 80% confluent. They were transfected in triplicates using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfections were performed for 5 hours at a DNA to lipofectamine ratio of 1:3 in MEM-EBSS lacking FBS. The transfection cocktail was mixed as follows: 50 ng of the pCMVβ β-galactosidase normalizing reporter plasmid (Clonetech, Mountain view, CA), 400 ng of pT81 (American Type Culture Collection, Manassas, VA) plasmid expressing firefly luciferase reporter driven by the androgen-dependent Probasin promoter. 800 ng of the pCI-Neo plasmid (Promega, Madison,WI) expressing human AR, and 800 ng of the pCI-Neo plasmid expressing hFKBP52, DrFKBP52, and/or DrFKBP52 mutants, with 800 ng of pCI-Neo plasmid containing normalizing vector.

Twenty-four to thirty hours after transfection, medium was replaced with medium containing 10 pM DHT (or a range of doses, as appropriate). After approximately fourteen-sixteen hours of incubation with hormone, cells in each well were lysed using 100 µL mammalian protein extraction reagent (M-PER) (Pierce, Rockford, IL)

supplemented with Complete ethylenediaminetetraacetic acid (EDTA)-free Mini Protease Inhibitor (Roche, Manheim, Germany) and spun at 15,000 rpms in a 4*C microcentrifuge to remove impurities. Luciferase expression was quantified by mixing 40 µL cell lysate with 100 µL of luciferase assay reagent (Promega, Madison, WI) in a single well for each sample on a 96-well plate. β-galactosidase expression was quantified by adding 20 µL cell lysate with 100 µL of Gal Screen Reagent (Tropix, Bedford, MA). The 96-well plates were incubated at room temperature, followed by quantification of luminescence by a microplate luminometer (Luminoskan Ascent, Thermo Labsystems). Luminescence was measured in Relative Light Units (RLU). The transfection-efficiency normalized levels of reporter expression (luciferase RLU/ β-galactosidase RLU) were reported in graphical form using Graphpad Prism software and were all normalized to 100% expression.

The data shown in the composite promoter graph represent five independent experiments plus/minus standard deviation of at least two separate samples, and figures are composite graphs representing data from at least three independent experiments.

### 3.2.6 Western Blot

Yeast cells growing overnight were diluted to an $OD_{600}$ of approximately 0.2 and grown until the $OD_{600}$ reached 0.8. For Western blot to detect receptors, which tend to precipitate in yeast lysates, yeast cells were pelleted, resuspended in extract buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 5% glycerol supplemented with protease inhibitors] and vortexed vigorously in the presence of glass beads. Lysates were then clarified at 15,000 rpm for 20 min at $4^0$C. For mammalian cell lysis, cells were washed with 1X PBS

and lysed with mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL) supplemented with protease inhibitors (Complete mini EDTA-free; Roche, Indianapolis, IN). Protein concentrations for both yeast and mammalian extracts were determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL). Typically 40 µg of total cellular protein was separated on a 10-20% Criterion gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes. The following antibodies were used: rabbit α-human SGTα (Protein Tech Group, Chicago, IL), mouse monoclonal α-FKBP52 (HI52B, HI52C, HI52D), rabbit α-human AR (Santa Cruz Biotechnology, Santa Cruz, CA ), and rabbit α-human GR (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies detecting yeast ribosomal protein L3 (a gift from Jonathan Warner, Albert Einstein College of Medicine) and Glyceraldehyde-3-phosphate dehydrogenase (6C5; Biodesign International, Saco, MN) were used as loading controls. The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies and bands were visualized with Immune-Star AP substrate (Bio-Rad, Hercules, CA) and exposed to X-ray film.

## 3.3 RESULTS

### 3.3.1 Designing and Optimizing the Gain-of-Function Random Mutagenesis Screening Strain

As stated previously comparative analysis is a very powerful tool for the identification of relevant functional domains and/or residues. Riggs et al. utilized random mutagenesis to identify the proline-rich loop region that overhangs the catalytic pocket on hFKBP52 as being critical for FKBP52 function (6). This same proline-rich loop is present in DrFKBP52, so we used this method to identify additional residues

necessary for hFKBP52 activity. Our thorough assessment of DrFKBP52 led us to the conclusion that it would be an excellent model protein for gain-of-function comparative analysis with hFKBP52 (Section 2.3.1).

Our initial experimental model for the random mutagenesis was based on the protocol used by Riggs et. al. (6). However, after a year of false positives, failed experiments, and inconsistent data, it was determined that we had to completely re-optimize this protocol for our needs. We re-created the screening strain, as it was a major contributor to inconsistent data. We also optimized hormone and 3AT concentration, which distinguishes hormone-dependent growth efficiency. These optimizations were essential in making this experiment model a success and are discussed further.

Our first step was to regenerate a random mutagenesis screening yeast strain containing an androgen receptor-mediated *HIS3*-selectable reporter (Figure 3.3). Thus, in the presence of hFKBP52-potentiated receptor activity the strain could grow robustly on histidine-lacking medium. However, in the presence of DrFKBP52, which does not potentiate receptor activity, the strain would grow very poorly on histidine-lacking medium. Only in the presence of of the gain-of-function DrFKBP52 mutants could the strain grow robustly on histidine-lacking medium in the absence of hFKBP52. The selection strain YNK435 (*MAT*a *ura3-52 lys2-801 ade2- 101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 pdr5*::*GT3Z his3*::*GT3H*) was transformed with AR-P723S, hFKBP52, DrFKBP52, or vector. hFKBP52 was used as a positive growth control, and vector as the negative growth control. We then used this newly transformed strain to identify the optimal DHT concentration that allowed for growth of the hFKBP52 strain, but minimal to no growth of

vector and DrFKBP52 strains. We added 1 nM, 5 nM, 10 nM and 20 nM DHT to selective media plates lacking histidine and titrated cell density for each sample at 100, 500, 1000, 5000, 10000 cells/spot (Figure 3.1). Spot tests indicated that 5 nM or 10 nM was sufficient to distinguish hormone-dependent growth between the hFKBP52 and DrFKBP52 strains. Because robust growth of hFKBP52 cells were seen on selective media plates containing 10 nM DHT, this dose was chosen.

In an effort to control for leaky *HIS3* gene expression, and to reduce vector and DrFKBP52 background, we optimized 3-amino-1,2,4-triazole (3-AT) concentrations. The reagent 3-AT was used because it is a competitive inhibitor of the *HIS3* gene product, imidazoleglycerol-phosphate dehydratase. Imidazoleglycerol-phosphate dehydratase is an enzyme catalyzing the sixth step of histidine production. Spot tests were done using 5, 10, 15, and 20 mM 3-AT at 10 nM DHT on selective media plates lacking histidine. Each strain was spotted at 100, 500, 1000, 5000, and 10000 cells/spot (Figure 3.2). We determined that 10 mM 3-AT is sufficient to suppress vector and DrFKBP52 background growth, yet would not be toxic to yeast.

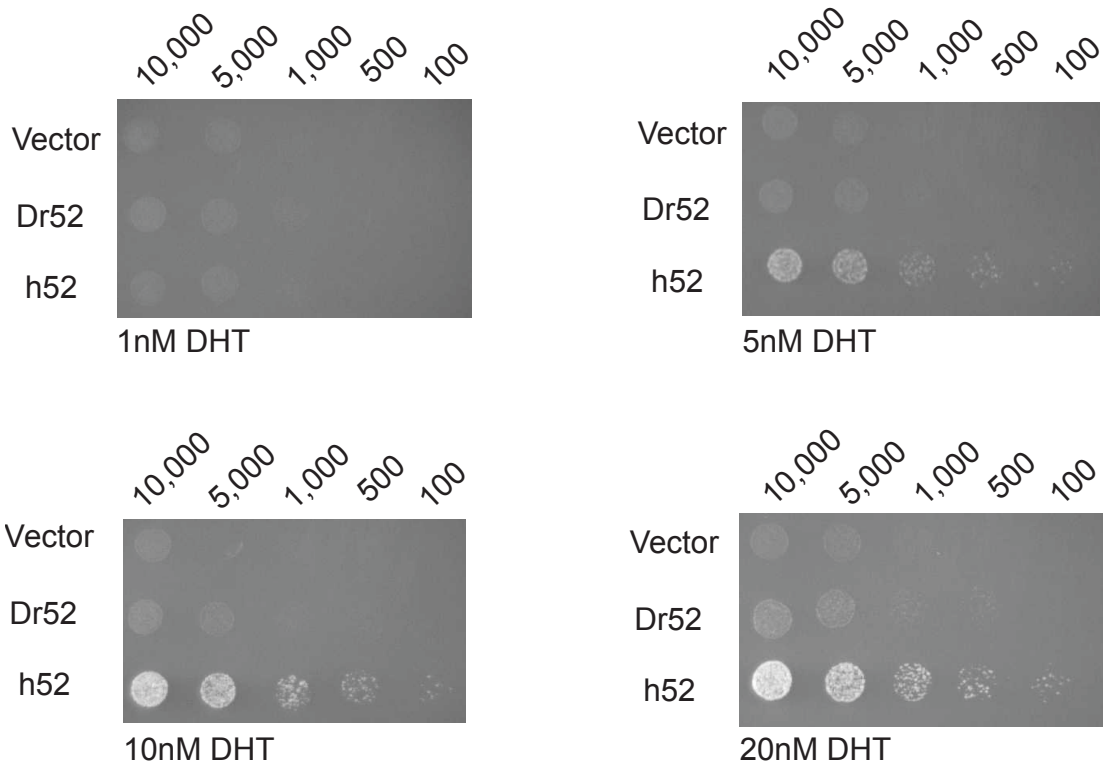



**Figure 3.1: Dihydrotestosterone Optimization for Random Mutant Screening.**

Various DHT concentrations were selected and hormone dependent cell growth was assessed. Cells were grown on SC-LW Plates containing hormone and 5 mM 3-AT. Spot test were performed at 10,000, 5,000, 1,000, 500, and 100 cells/spot.

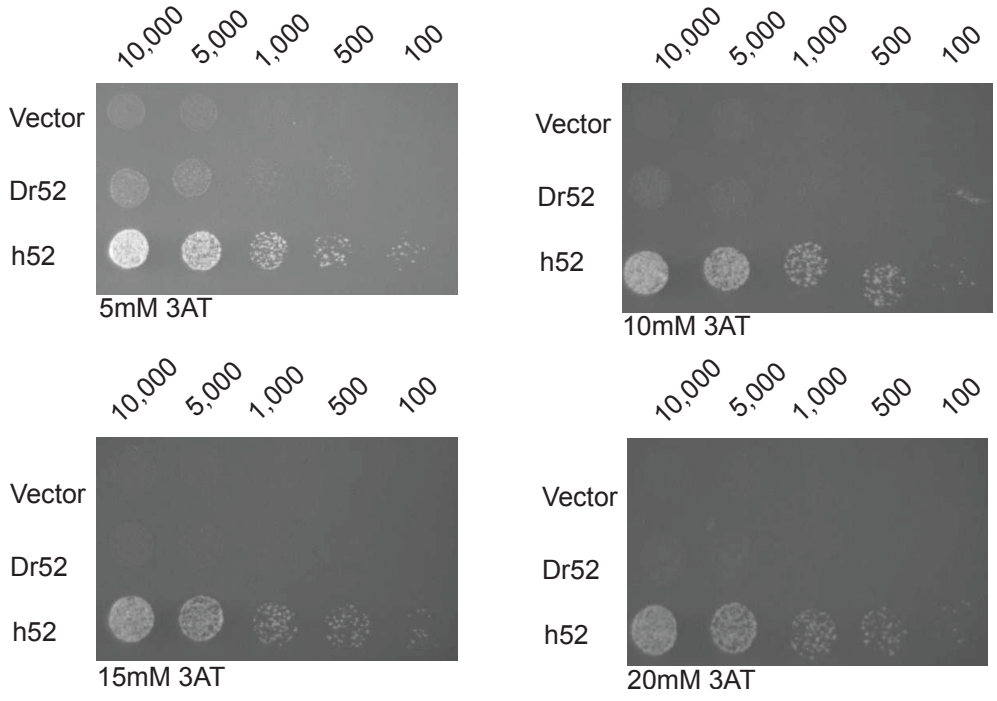



**Figure 3.2: 3-AT Optimization for Random Mutant ScreeningVarious**

3-AT concentrations were assessed for the ability to suppress leaky expression of the *HIS3* reporter. Cells were grown on SC-LW Plates+10nM DHT+3AT. Spot test were performed at 10,000, 5,000, 1,000, 500, and 100 cells/spot.

Our next step was to remove the DrFKBP52 expression plasmid from our selection strain, through replica plating, in order to start with a DrFKBP52 negative background in which to put the DrFKBP52 random mutants. In this method, a plasmid-bearing yeast strain is grown non-selectively twice overnight (to allow loss of the plasmid). Cells are than plated at a very low density on selective media plates. Colonies lacking the plasmid are identified by replica plating onto selective media plates lacking the specific nutritional supplement encoded by the marker gene on the plasmid of interest. If the plasmid is lost those colonies would not grow on media lacking the selective nutritional supplement. We were able to identify five isolates from the replica plating. We selected three of the isolates to use for a functional yeast assay to determine which strains would be used for random mutagenesis. Vector, hFKBP52, and DrFKBP52 expression plasmids were co-transformed into each strain and incubated for 4 days at 30°C. Three isolates were selected from each plate and assayed for functional competency. Human FKBP52 and vector isolates were kept as hormone-dependent growth controls for random mutagenesis screening.

### 3.3.2 Genetic Screen for Danio rerio FKBP52 Random Mutants that Up-Regulate AR-P723S Activity

A plausible explanation for the functional diversity between hFKBP52 and DrFKBP52 is that DrFKBP52 likely lacks critical residues necessary for productive interaction with the steroid hormone receptors. In this study such residues were selected for, through yeast genetics, by evaluating gain-of-function mutations in DrFKBP52 that allow it to potentiate the hormone signaling pathway in a manner similar to that of hFKBP52. The yeast selection strain described in section 3.3.1 was used for

these studies. This strain is unique in that its genome has been manipulated to express HRE:*HIS3* and an HRE:LacZ reporter gene. The *HIS3* gene is attached to a weak promoter having an adjacent upstream hormone response element (Fig. 3.3). A *HIS3* inhibitor, 3-AT, was added to the growth medium, to control for leaky expression of the *HIS3* construct in the absence of hormone. This strain stably expresses AR and medium-lacking histidine was dependent upon the ligand dihydrotestosterone (DHT). This allowed for us to have selectivity bases on growth, which was equivalent to hormone binding and receptor activity. Figure 3.3 shows robust growth in yeast cells expressing hFKBP52, in medium supplemented with DHT. While little or no growth was detected in yeast expressing DrFKBP52 or Vector. This strain also expressed AR P723S, a point mutant, which is hyperdependent on FKBP52 for hormone-induced activity.

To generate DrFKBP52 gain-of-function mutants that support DHT-dependent yeast growth, we utilized the random mutagenesis method previously used in the FKBP51 study (6). Random mutagenesis was optimized to produce three to five mutations per PCR product. PCR products were gel purified and co-transformed with linearized vector into yeast cells. Transformed product was plated on the selective growth medium and incubated until distinct colonies appeared, about 1 week. As a control, vector, DrFKBP52, and hFKBP52 were also grown on separate plates, to adequately assess DHT-dependent yeast growth. In addition, the hormone-responsive β-galactosidase reporter gene was independently utilized to confirm and quantify DHT-induced β-galactosidase activity in liquid culture.

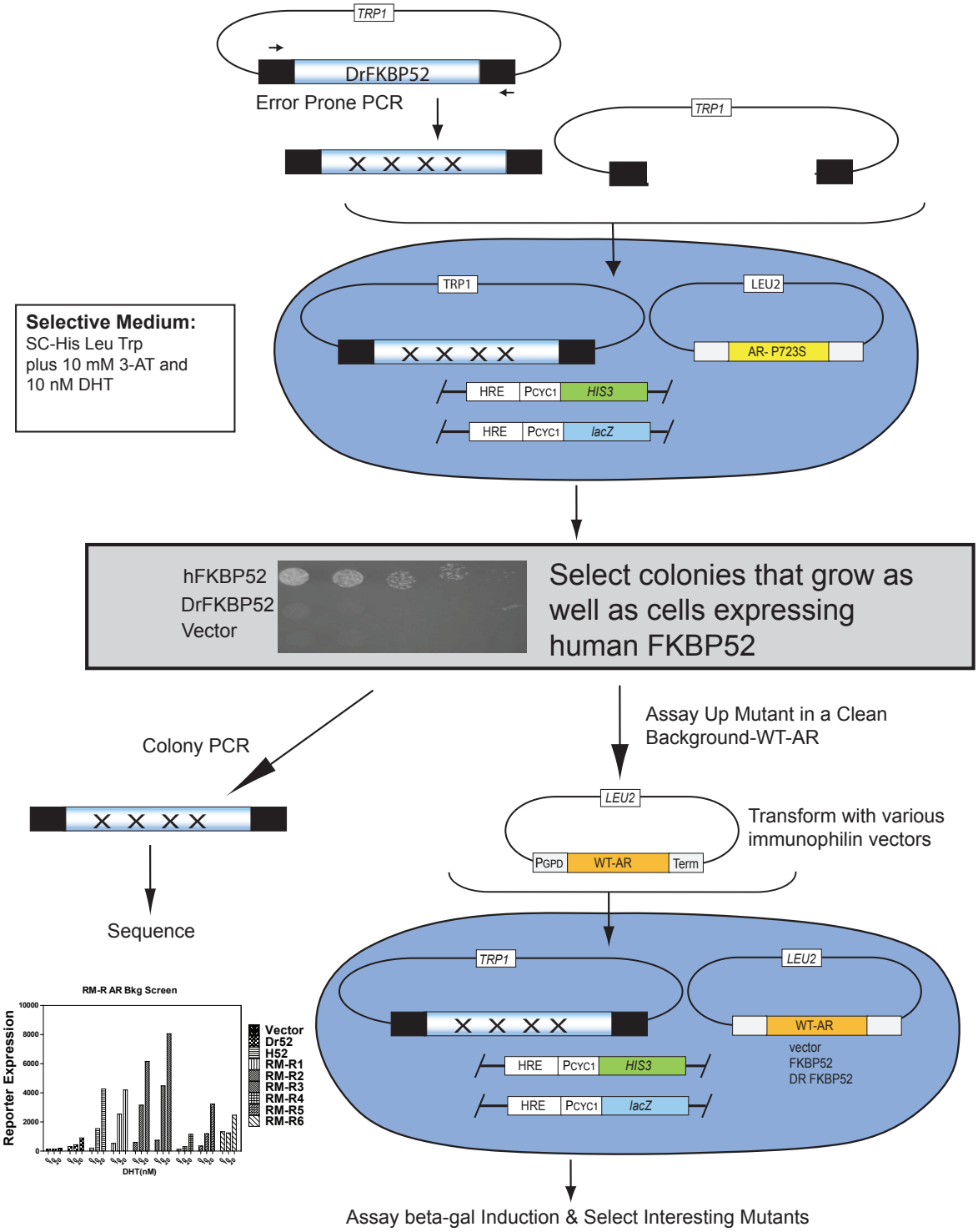



**Figure 3.3: Scheme for the Identification of Danio rerio FKBP52 Random Mutants that Up-Regulate the Phenotype of the AR-P723S.**

Figure 3.3: Scheme for the Identification of Danio rerio FKBP52 Random Mutants that Up-Regulate the Phenotype of the AR-P723S.

(A). Libraries of random *Danio rerio* FKBP52 mutants were independently generated by error- prone PCR using primers binding upstream in the vector yeast GAPDH promoter (PGPD) or downstream in the transcriptional terminator (Term). Mutant libraries were co-transformed with a linearized vector such that homologous recombination between the common promoter and terminator regions on these fragments (about 100 nucleotides each) reconstitutes a *TRP1*- marked Danio rerio FKBP52 expression plasmid. The parental strain harbors a *LEU2*-marked AR- P723S expression plasmid and integrated *HIS3* and *lacZ* reporter genes driven by hormone- responsive promoter element (HRE). Transformants were plated on selective growth medium plates supplemented with 10mM 3-amino-1, 2, 4-triazole and 10nM DHT. To confirm that growth was dependent on mutant DrFKBP52 a liquid assay was performed using parent strain expressing AR-P723S and laZ reporter plasmid. Identified up-mutants, exhibiting the gain-of- function phenotype, were extracted from yeast and co-transformed into secondary strains expressing WT-AR and laZ reporter plasmid. The *Danio rerio* FKBP52 mutant genes were sequenced to identify relevant mutations. (B) Yeast strains containing a hormone-inducible *HIS3* gene and expressing AR plus either Vector, hFKBP52 or/and *Danio rerio* FKBP52 were grown on selection medium containing a growth-limiting concentration of 10nM DHT, as described in Materials and Methods. Yeast grows well only in the presence of FKBP52, which potentiates AR activity.

To ensure that DrFKBP52 mutant potentiation was not yeast strain-dependent, the mutant DrFKBP52 gene was transferred into a clean background strain transformed

with a different hormone-inducible β-galactosidase reporter and wild type AR or AR-P723S. If the mutants maintained their ability to augment the receptor activity, the mutant gene was sequenced.

Mutants were isolated from numerous independent error-prone PCR reactions, with the expectation that key mutations would repeatedly arise. A total of 34 gain-of-function mutants were isolated from twelve independent libraries; of these, 12 had mutations in FK1:  F49S and A116V/T, and 9 were located in the FK2 domain the T157R/A, and S172Y (Figure 3.4). Each was isolated multiple times from independent libraries. The DrFKBP52 A111 is analogous to hFKBP52 A116 and, although the residues are the same in both proteins, previous studies comparing FKBP51 suggested that changing this alanine to a valine in FKBP51 also conferred gain-of-function to FKBP51 (6) Random mutant S172Y was also previously evaluated for potentiation activity, but no effects were seen (Figure 2.7), However, residues F49, T157 do show functional sequence divergence and maybe important for receptor activity.

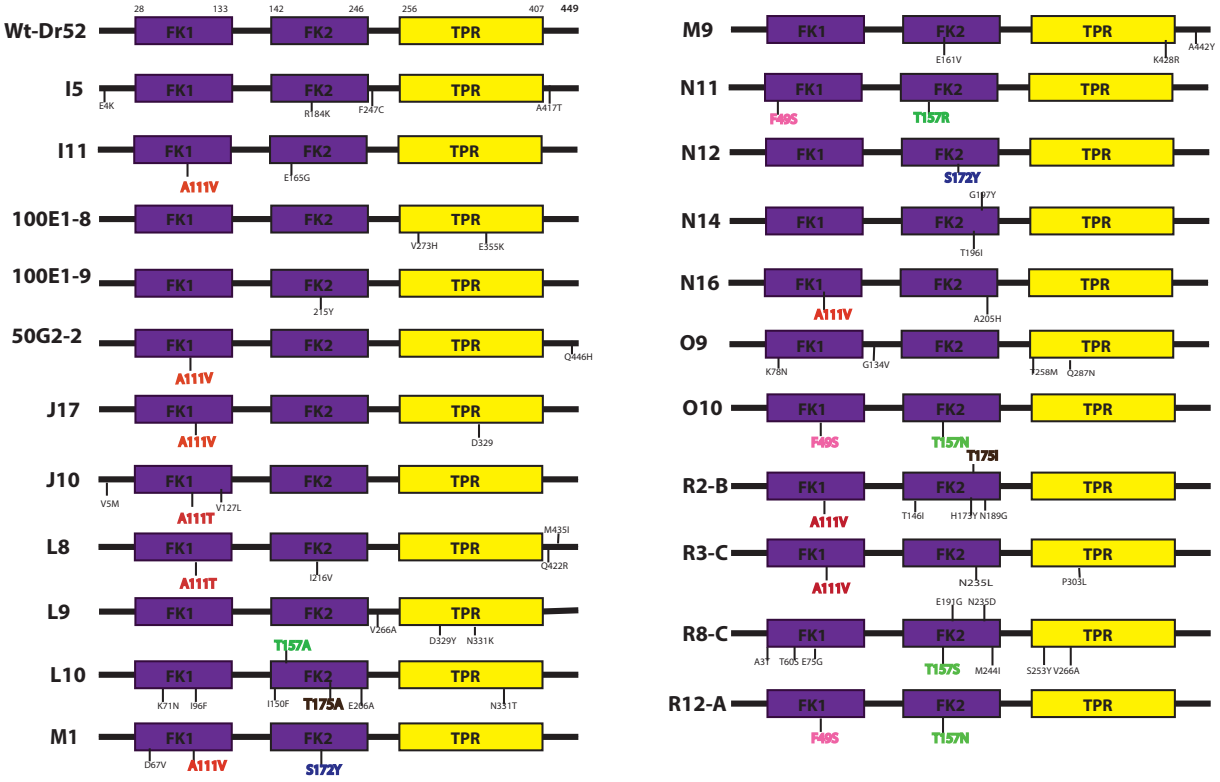



**Figure 3.4: Danio rerio FKBP52 Potentiating Mutants Isolated in the Selection Strain.**

The top map represents the domain arrangement of DrFKBP52 in a linear manner with amino acid numbering at the domain boundaries. Isolated mutants are aligned below. Each mutant is identified by the PCR round from which it was isolated (E to R) and an isolate number. Repeatedly identified mutations of particular interest are shown in bold and underlined.

### 3.3.3 Characterization of DrFKBP52 Mutants

To confirm the individual role of each identified mutant, site-directed mutagenesis was utilized to generate the corresponding point mutant in wild type DrFKBP52. Mutants were analyzed in yeast expressing either wild type AR or AR-P723S. The residues at position 111 and 157 are critical for DrFKBP52 potentiation as the A111V and T157R DrFKBP52 mutants were able to potentiate AR signaling approximately 3- to 5-fold that of wild type DrFKBP52 when co-expressed with wild type AR (Figure 3.5A). However, AR-P723S showed only a 2-fold enhancement. Remarkably, when A111V and T157R are combined DrFKBP52 potentiated signaling comparable to hFKBP52 alone, in both wild type AR and AR-P723S strains (Figure 3.5A and B). A similar analysis was performed in 52KO MEF cells, where similar results were obtained (Figure 3.5C). The mutant F49S was co-expressed with wild type AR, but we did not observe any significant regulation of receptor function as compared to wild type DrFKBP52. Western blot analysis indicated equal FKBP expression levels and stable expression levels of receptor proteins.

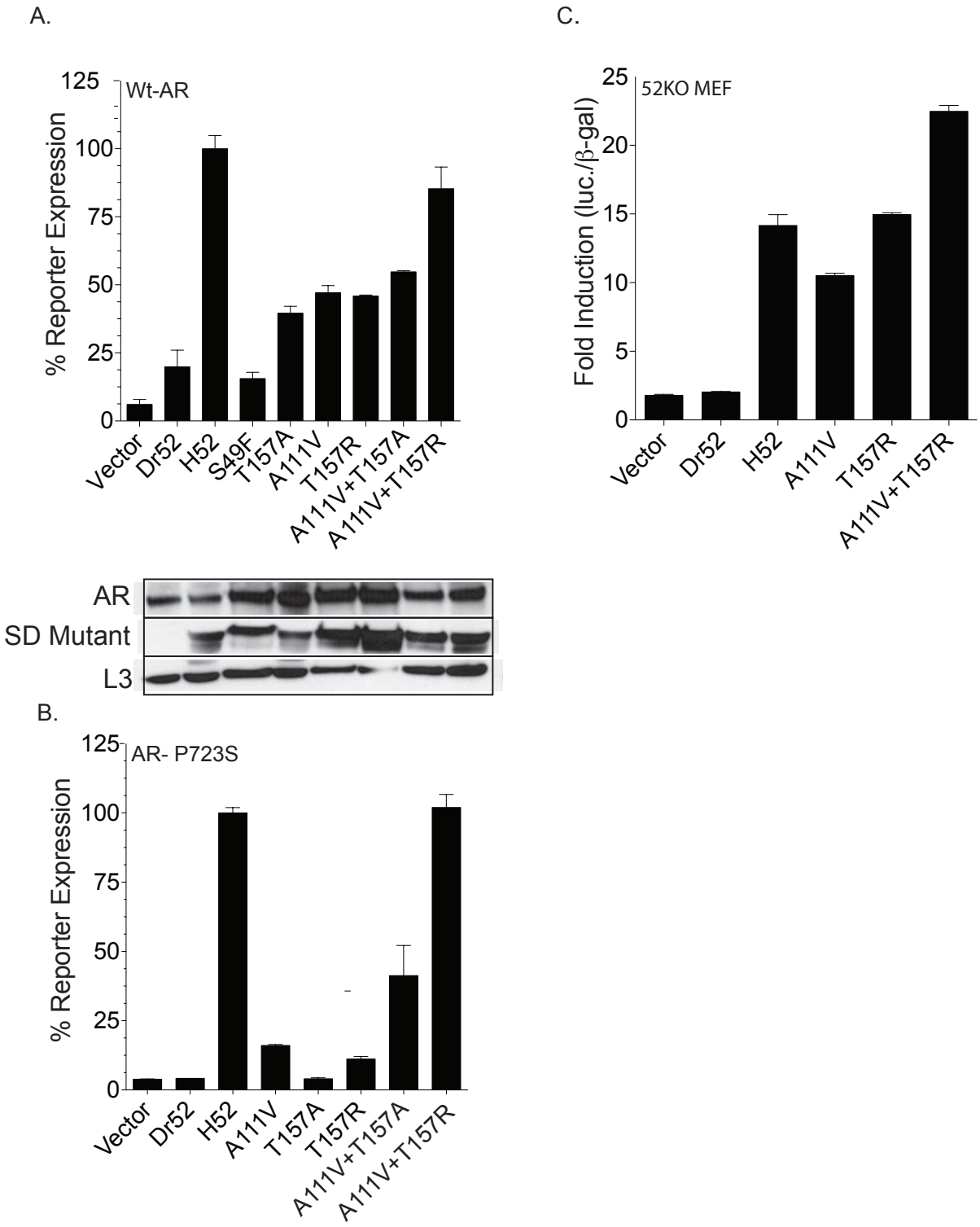



**Figure 3.5: Functional Analysis of Amino Acids at Positions 49, 111, and 157.**

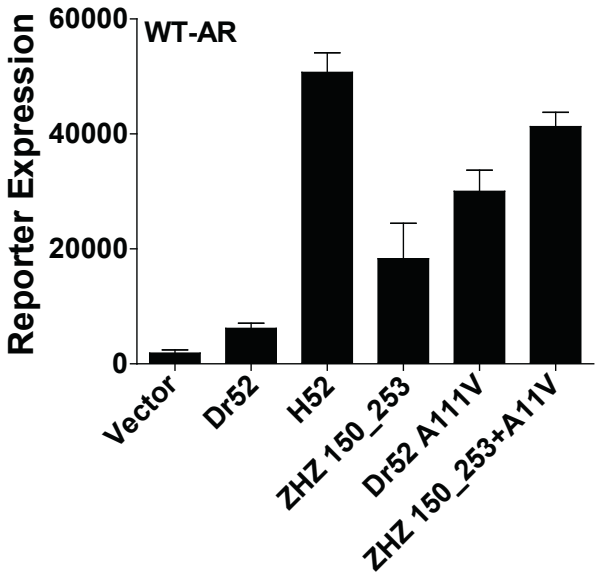
Figure 3.5: Functional Analysis of Amino Acids at Positions 49, 111, and 157.

(A) Hormone-dependent reporter gene activity was measured in yeast strains expressing wild- type AR (3nM DHT) and AR-P723S (10nM DHT) plus the indicated FKBP. The single mutants (right) and double mutants (left) were assayed for activity. Relevant single and double mutants were assayed in FKBP52 hyper-dependent AR mutant P723S (bottom). Protein expression levels were monitored by Western immunostaining for the introduced AR- P723S, FKBP and the endogenous ribosomal subunit L3. (B) FKBP activities were similarly determined in transfected 52KO MEF cells expressing wild-type AR and a hormone-dependent luciferase reporter. Protein expression levels were monitored by Western immunostaining for the introduced FKBP, AR, and endogenous GAPDH.

### 3.3.4 Characterization of Chimera ZHZ-150_253 in Combination With A111V

These results indicate that A111 in DrFKBP52 has a role in the ability to potentiate AR signaling. We used Chimera ZHZ-150_253 to map protein regions that influence potentiation and further evaluate the role of the FK2 domain. In previous studies, the FK1 domain appears to be the sole difference with respect to potentiation of AR. Chimera ZHZ-150_253 contains the DrFKBP52 FK1 and TRP domains and the hFKBP52 FK2 domain. When Chimera ZHZ-150_253 is combined with A111V it results in levels of potentiation equivalent to that observed with hFKBP52 in both wild type AR and AR-P723S (Figure 3.6). Separately, Chimera ZHZ-150_253 and A111V had intermediate levels of potentiation. Results suggest full activity differences between hFKBP52 and DrFKBP52 are to be attributed to both the proline-rich loop within FK1 and the FK2 domain.

A.




B.

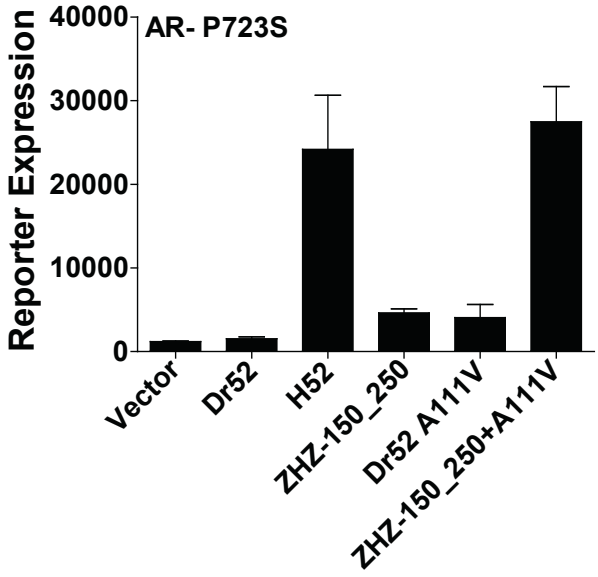



**Figure 3.6: Functional Analysis of Chimera ZHZ-150_253 with A111V Mutation.**

Figure 3.6: Functional Analysis of Chimera ZHZ-150_253 with A111V Mutation.

(A) Hormone-dependent reporter gene activity was measured in yeast strains expressing wild- type AR at 3nM DHT and the indicated Chimera and Site-directed mutant FKBP. (B) FKBPs activities were similarly determined in AR-P723S at 10nM DHT. Protein expression levels were monitored by Western immunostaining for the introduced wt-AR, AR- P723S, FKBPs and the endogenous ribosomal subunit L3.

### 3.3.5 Mutation of DrFKBP52 A111 AND FKBP52 A116

Although this analogous residue has been identified in a previous study (6), the significance of this residue was not fully understood. This residue is positioned on the periphery of the proline-rich loop region. Here we have attempted to uncover its importance in hFKBP52 activity. We have done this through evaluating the effects of different amino acids residues at this position in both FKBPs. These mutations were introduced by site-directed mutagenesis and co-transformed into yeast expressing wild type AR or AR-P723S. It was observed that hydrophobic residues valine, lysine, and tyrosine have the greatest ability to enhance receptor function with valine having maximal potentiation, in both FKBPs (Figure 3.7). Negativly charged amino acids, glutamic acid and aspartic acid, drastically decrease activity to approximately 50% that of hFKBP52 and no effects are seen in DrFKBP52. A moderate decrease is also seen with Arginine for both FKBPs in the wild type AR strain (Figure 3.7A). Although AR-P723S drastically shows the divergence in activity between the amino acids, the trend is consistent. It should be noted that we observe a 40% increase in potentiation with the hFKBP52-A116V mutation in the regulation of AR-P723S (Figure 3.7C). The identical trend in amino acid modulation between hFKBP52 and DrFKBP52 strengthen the evidence that this residue has importance in the proline-rich loop region and interaction with the receptor, specifically the BF3 surface.

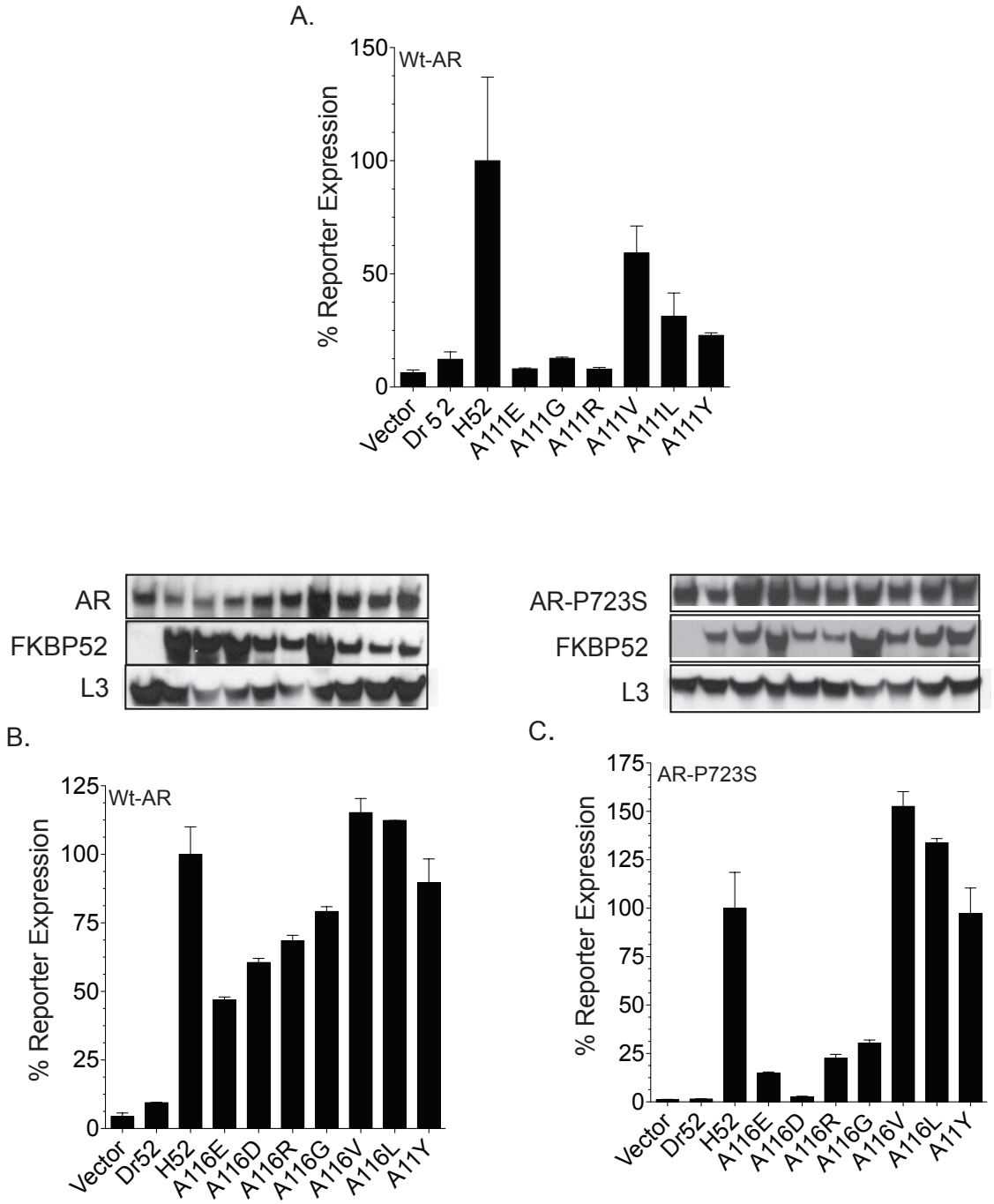



**Figure 3.7: Functional Modulation of FKBP A111 and A116 Mutations.**

Figure 3.7: Functional Modulation of FKBP A111 and A116 mutation

(A). AR signaling and FKBP expression was assayed in yeast, DrFKBP52: A111V and hFKBP52: A116V or (C) AR:P723S, only hFKBP52: A116V. Hormone-dependent reporter gene activity was measured in yeast strains expressing wild-type AR (3nM DHT) and Dr52 FKBP Site- directed mutants (B) Human FKBP52 FKBP Site-directed. (C) Relevant hFKBP52 single mutants were assayed in hFKBP52 and hyper-dependent AR:P723S (10nM DHT). Protein expression levels were monitored by Western immunostaining for the introduced AR- P723S, FKBP and the endogenous ribosomal subunit L3.

### 3.3.6 Mapping Other Relevant Residues in the FK2 Domain

Our results indicate a role for the FKBP52 FK2 domain in the potentiation of steroid hormone receptor signaling, yet specific residues are unknown. DrFKBP52-T157R has the capacity to enhance AR function, so we propose that hFKBP52 activity could be conveyed by the arginine-rich region near the FK linker (Figure 3.8A). It should be noted that converse mutation in hFKBP52 had no effect on receptor activity. However, this arginine-rich region is located 15 amino acids away from this residue. Crystal structure shows that these residues are on the surface of the protein. The hFKBP52 and DrFKBP52 crystal structures were superimposed to determine orientation of residues (Figure 3.8B). We used site-directed mutagenesis to generate hFKBP52-R152A, -R153A, and -R157A expressions plasmids, which were then co-expressed with wild type AR and functional yeast assays were performed to assessed activity. Functional yeast assays determined that all three mutants significantly abrogated hFKBP52 ability to potentiate wt-AR activity (Figure 3.8C). These arginine residues are positively charged, allowing the side chains to participate in protein-protein interactions and ionic bonding, which could be important for interaction with the FK1 domain or receptor.

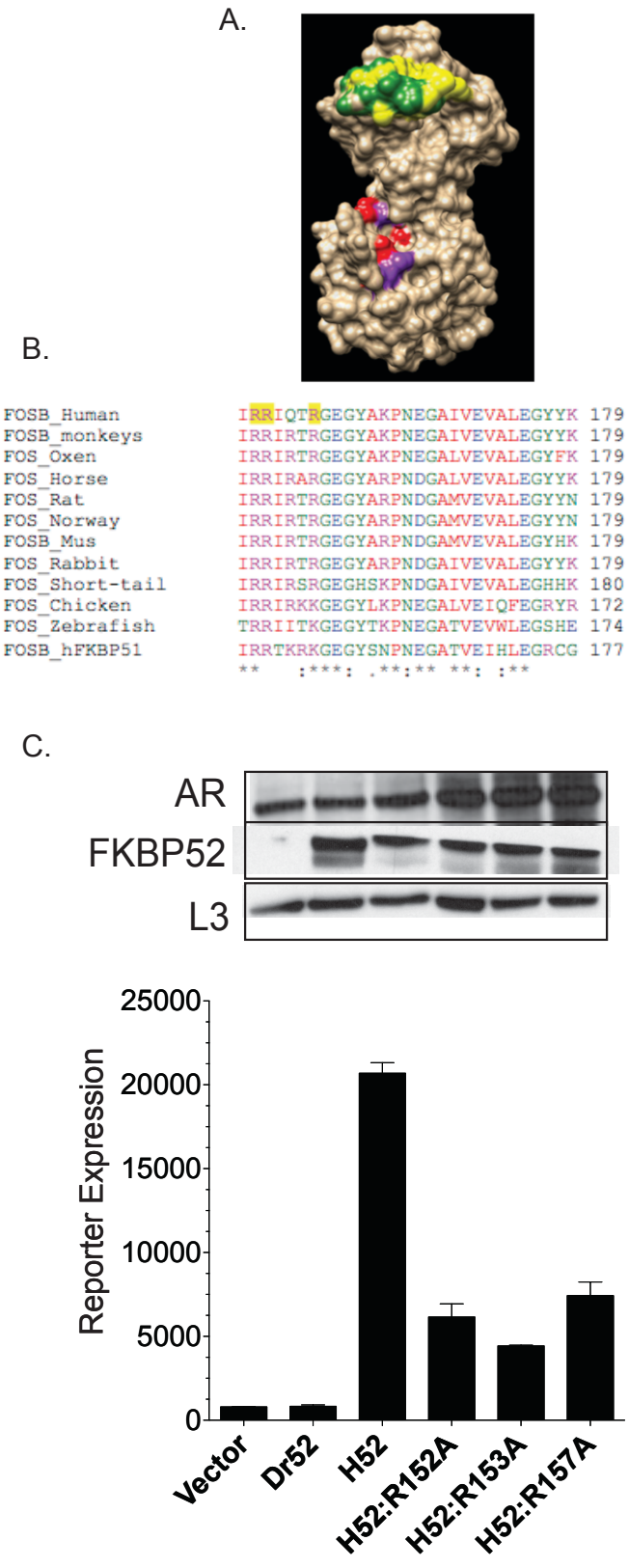



**Figure 3.8: Functional Analysis of Arginine Motif**

Figure 3.8: Functional Analysis of Arginine Motif

(A) hFKBP52 and DrFKBP52 FK1-FK2 domains were superimposed to determine placement of Arginine in relation to the orientation of the proline-rich loop. hFKBP52 (green and red) and DrFKBP52 (yellow and purple). (B) ClustalW2 alignment shows residue conservation. (C) Hormone-dependent reporter gene activity was measured in yeast strains expressing wild-type AR at 3nM DHT and the indicated Site-directed mutant FKBP. Protein expression levels were monitored by Western immunostaining for the introduced wt-AR, AR- P723S, FKBPs and the endogenous ribosomal subunit L3.

## 3.4 DISCUSSION

Human FKBP52 is a known positive regulator of hormone-dependent transcriptional activity of steroid hormone receptors. Its physiological relevance has been established in male and female mice FKBP52 knockout results in reproductive abnormalities. The mechanistic basis for receptor potentiation is still poorly understood, however, recent studies implicate its presence at the transcription level (107). It is known that FKBP52 assembles with receptor complexes through association with Hsp90, and exerts its effect on the receptor activity. In particular the N-terminal, FK1 PPIase domain of hFKBP52 has been recognized as important for activity, but not sufficient for full activity. The FK1 domain importance is further established by the lack of receptor potentiation in the presence of the FK506 ligand, which binds the PPIase pocket (25). Further evidence was obtained in a previous study comparing the highly similar FKBP51 protein. Gain-of-function random mutagenesis determined that the double A116V and L119P mutation enable the nonfunctional FKBP51 to potentiate receptor activity similar to that of FKBP52 . Converse mutations identified that the P119L mutation reduced receptor function by 40%, leading to the conclusion that the proline-rich loop region is critical for receptor potentiation. However, the significance of the A116V mutation was still unclear and the FK2 domain unrecognized (6). However, previous findings implicate the FK2 domain as having a role in potentiation (25, 109, 113).

Despite the presence of the proline-rich loop, CKII site, Hsp90 binding motif, and many other similarities to hFKBP52, DrFKBP52 does not potentiate receptor activity. DrFKBP52 was randomly mutated, and a yeast genetic screen created to isolate

DrFKBP52 mutants that gain potentiation activity (Figure 3.3). The gain-of-function mutants that were repeatedly identified were A111V (9 independent identifications) and T157R (7 independent identifications) (Figure 3.4). Separately, these mutants had sufficient activity to increase potentiation of wild type AR three to five-fold, as compared to DrFKBP52 (Figure 3.5). Remarkably, the DrFKBP52-A111V/T157R double mutant completely restored activity similar to that of hFKBP52 in yeast and mammalian cells (Figure 3.5). DrFKBP52-F49S and -S172Y did not confer potentiation in any of the yeast strains. It should be noted that the T157A mutant was able to confer activity separately, however, the double mutant A111V/T157A did not function like hFKBP52 or its counterpart DrFKBP52-A111V/T157R; implying a specific possible interaction between the FK1 and FK2 domains or specific, but separate contact with the receptor. Though converse mutation in hFKBP52 did not affect activity, the reoccurrence of residue A116 in this study implicates it again as an important residue. Since DrFKBP52 and hFKBP52 both naturally contain alanine at positions 111 and 116 respectively, we tested whether modulating these residues would have potentiation activity exceeding that of wild-type hFKBP52 (Figure 3.7B and C). The hydrophobic residues at this position in DrFKBP52 produced only a small increase in potentiation of wild type AR, while the positive and negative residues significantly reduced potentiation ability. This was further corroborated with AR-P723S where a dramatic increase in potentiation was seen with the hydrophobic residues and a drastic reduction of potentiation was seen with positive and negative residues (Figure 3.7C). The same trend was seen in DrFKBP52 (Figure 3.7A). These results highlight the importance of the residue, however, its significance was still unclear.

We have also indicated a plausible important region located upstream of FK1 linker region. We presume that the FK2 domain role has been firmly established through chimera data, but its relevance is still unclear. The DrFKBP52-T157R mutant is very efficient in enhancing receptor function, yet the converse mutation in hFKBP52 had no significant effects (Data not shown). Interestingly, this mutation is located down-stream of a highly conserved stretch of residues (IRRIQTR), a positively charged and hydrophobic region. Like the proline-rich loop region it is highly conserved between DrFKBP52, FKBP52 and FKBP51 species. We have determined that changing any of these arginines to alanine in hFKBP52 abrogates potentiation by up to 60% (Figure 3.8). Because this region is highly conserved it could be necessary for conformational integrity like position A116 and not strictly a sequence difference. Though we have not confirmed a specific residue in the FK2 domain that is important for hFKBP52's potentiation, we postulate that it may be near the FK1 linker region and may facilitate a specific interaction with the FK1 loop region. This is suggested by the DrFKBP52 double mutant can potentiate the receptor like hFKBP52. This is exciting because we have established another possible target for inhibitor design.

# CHAPTER 4: FKBP HOMOLOGY MODELING

## 4.1 RATIONALE

Our observation in Aim 3 revealed a positive regulatory role for position DrFKBP52-A111 and hFKBP52-A116. The analogous residue was also identified as having a positive affect in FKBP51 regulation of steroid hormone receptor signaling. The reoccurrence of this mutation indicates that it could be an important residue in FKBP52 activity. Because biochemical studies alone do not convey the structural significance as it relates to function we have utilized structural studies to further elute the importance of this residue. To resolve the structural changes in DrFKBP52-A111V that contribute to the gain in activity we used homology modeling.

Homology modeling, also known as comparative modeling, is a method in which the atomic-resolution model of a target, “unknown” protein is formed using an experimentally resolved three-dimensional structure of a homologous protein “template”. Many of the experimental method use to resolve 3D structure such as X-ray crystallography and protein NMR are complex and time-consuming. Homology modeling provides useful structural models for generating a hypotheses about a protein's function and directing further experimentation. It is also useful in reaching qualitative conclusions about the biochemistry of the target sequence, especially in understanding why certain residues are conserved (116). Though evolutionarily related proteins change in amino acid sequence, the overall three-dimensional protein structure is evolutionarily more conserved than would be expected on the basis of sequence conservation. For this fact we used hFKBP52 and hFKBP51 for homology modeling with DrFKBP52 and DrFKBP52-A111V.

We hypothesize that a comparative structural analysis of hFKBP52, hFKBP51, DrFKBP52 and DrFKBP52-A111V will reveal key structural elements that contribute to FKBP52 activity. Because this point mutant is near the proline-rich loop, it could also be helpful in determining the surface area important for protein-protein interaction.

## 4.2 MATERIAL AND METHODS

### 4.2.1 Template Recognition and Initial Alignment

We used NCBI:Pubmed for template and target proteins selection and extraction (http://www.ncbi.nlm.nih.gov/pubmed). Basic Local Alignment Search Tool (BLAST) was used to determine template sequence homology by specific parameters. The quality of the homology model is dependent on the quality of the sequence alignment and template structure. The approach can be complicated by the presence of alignment gaps that indicate a structural region present in the target but not in the template. Model quality also declines with decreasing sequence identity. Sequence alignment tool is necessary to adequately evaluate homology-modeling compatibility for template and target.

### 4.2.2 Alignment Correlation

To create the model we need a sequence alignment file between our target and the selected template sequences of FKBP51, DrFKBP52, FKBP52 and DrFKBP52-A111V. For this aim, we will use another online server, the multiple sequence alignment ClustalW2. The sequence for the selected template can be obtained directly from the BLAST webpage or from the PDB website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

### 4.2.3 I-TASSER Homology Modeling

DrFKBP52 and DrFKBP52-A111V amino acid sequence was submit to I-TASSER for homology modeling (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Results were then individually assessed using molecular modeling software, MacPyMOL.

## 4.3 RESULTS

### 4.3.1 FKBP Sequence Alignments

BLAST and ClustalW2 sequence alignment indicates that both FKBP51 (Figure 4.2 and 4.3) and FKBP52 (Figure 2.1 and 4.1) are suitable templates to render high quality homology modeling. In both template alignment models the gaps are 0%, the absence of sequence gaps is important for accurate predictions of the structural loop. The Expect-value (E-value) is less than 1, this is the number that gives the average for false positives, which is a measure of alignment reliability. The E-value of FKBP51 and FKBP52 alignment with DrFKBP52 E-values is close to 0 making them reliable matches. Their sequence identity is also above 50%, which make each template optimal for comparative modeling (Figure 4.1 and 4.2).

**PEPTIDYL-PROLYL CIS-TRANS ISOMERASE FKBP4 [HOMO SAPIENS]**
**ALIGNMENT STATISTICS FOR FKBP52 and DrFKBP52**

| Score | E-Val | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 611 bits (1575) | 0.0 | Compositional matrix adjust. | 272/419:65% | 361/419:86% | 0/419:0% |

```
h52    1    MTAEEVVNEGCSIPIEGEDITPKKDGGVLKLVKKEGTGTELPMIGDKVFVHYVGTLLDGS  60
            M A E   +   +P+EG DI+PK+D GVLK++K+EGTGTE+PMIGD+VFVHY G LLDG+
Dr52   6    MKATESGAQSAPLPMEGVDISPKQDEGVLKVIKREGTGTEMPMIGDRVFVHYTGWLLDGT  65

h52    61   QFDSSRDRGEKFSFELGKGQVIKAWDIGVATMKIGEICQLTCKPEYAYGAAGSPPKIPPN  120
            +FDSS DR +KFSF+LGKG+VIKAWDI +ATMK+GE+C +TCKPEYAYG+AGSPPKIPPN
Dr52   66   KFDSSLDRKDKFSFDLGKGEVIKAWDIAIATMKVGEVCHITCKPEYAYGSAGSPPKIPPN  125

h52    121  ATLLFQVELFDFRGEDITDDEDGGITRRIITKGEGYTKPNEGATVEVWLEGSHEDRVFDE  180
            ATL+F+VELF+F+GED+T++EDGGI RRI T+GEGY KPNEGA VEV LEG ++D++FD+
Dr52   126  ATLVFEVELFEFKGEDLTEEEDGGIIRRIQTRGEGYAKPNEGAIVEVALEGYYKDKLFDQ  185

h52    181  RELKFEVGDGENLGLPLGVEKALQAMEQGEEALFTIKPKYGFGTAGSEKYNIPPNATLQY  240
            REL+FE+G+GENL  P G+E+A+Q ME+GE ++  +KP Y FG+ G EK+ IPPNA L+Y
Dr52   186  RELRFEIGEGENLDLPYGLERAIQRMEKGEHSIVYLKPSYAFGSVGKEKFQIPPNAELKY  245

Dr52   241  KIKMKAFEKAKESWEMNTIEKLEQSVIVKEKGTQYFKEGKYKQAIVQYKRIVSWLEHESS  300
            ++ +K+FEKAKESWEMN+ EKLEQS IVKE+GT YFKEGKYKQA++QYK+IVSWLE+ESS
Dr52   246  ELHLKSFEKAKESWEMNSEEKLEQSTIVKERGTVYFKEGKYKQALLQYKKIVSWLEYESS  305

h52    301  MQPDDEEKAKALRLAAYLNLAMCYLKLQDANPALENCDKALELDANNEKALFRRGEALVV  360
               ++ +KA+ALRLA++LNLAMC+LKLQ  + A+E+C+KALELD+NNEK LFRRGEA +
Dr52   306  FSNEEAQKAQALRLASHLNLAMCHLKLQAFSAAIESCNKALELDSNNEKGLFRRGEAHLA  365

h52    361  MKEFDMAKVDFQRVIELYPANKAAKSQISICQKHMREQHEKDKRLYANMFQKFAERDAK  419
            + +F++A+ DFQ+V++LYP NKAAK+Q+++CQ+ +R Q  ++K+LYANMF++ AE + K
Dr52   366  VNDFELARADFQKVLQLYPNNKAAKTQLAVCQQRIRRQLAREKKLYANMFERLAEEENK  424
```

**Figure 4.1: FKBP52 and DrFKBP52 BLAST Sequence Alignment**

Sequence was aligned using BLAST alignment software. Accession numbers: NP_002005 (FKBP52) and NP_958877 (DrFKBP52).

**PEPTIDYL-PROLYL CIS-TRANS ISOMERASE FKBP5 [HOMO SAPIENS]**
**ALIGNMENT STATISTICS FOR FKBP51 and DrFKBP52**

| Score | E-val | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 525 bits (1352) | 0.0 | Compositional matrix adjust. | 251/409:61% | 320/409:78% | 2/409:0% |

```
h51     17   TVAEQGEDITSKKDRGVLKIVKRVGNGEETPMIGDKVYVHYKGKLSNGKKFDSSHDRNEP  76
             ++  +GEDIT KKD GVLK+VK+ G G E PMIGDKV+VHY G L +G +FDSS DR E
Dr52    12   SIPIEGEDITPKKDGGVLKLVKKEGTGTELPMIGDKVFVHYVGTLLDGSQFDSSRDRGEK  71

h51     77   FVFSLGKGQVIKAWDIGVATMKKGEICHLLCKPEYAYGSAGSLPKIPSNATLFFEIELLD  136
             F F LGKGQVIKAWDIGVATMK GEIC L CKPEYAYG+AGS PKIP NATL F++EL D
Dr52    72   FSFELGKGQVIKAWDIGVATMKIGEICQLTCKPEYAYGAAGSPPKIPPNATLLFQVELFD  131

h51     137  FKGEDLF--EDGGIIRRTKRKGEGYSNPNEGATVEIHLEGRCGGRMFDCRDVAFTVGEGE  194
             F+GED+   EDGGI RR   KGEGY+ PNEGATVE+ LEG    R+FD R++ F VG+GE
Dr52    132  FRGEDITDDEDGGITRRIITKGEGYTKPNEGATVEVWLEGSHEDRVFDERELKFEVGDGE  191

h51     195  DHDIPIGIDKALEKMQREEQCILYLGPRYGFGEAGKPKFGIEPNAELIYEVTLKSFEKAK  254
             +  +P+G++KAL+ M++ E+ +  + P+YGFG AG  K+ I PNA L Y++ +K+FEKAK
Dr52    192  NLGLPLGVEKALQAMEQGEEALFTIKPKYGFGTAGSEKYNIPPNATLQYKIKMKAFEKAK  251

h51     255  ESWEMDTKEKLEQAAIVKEKGTVYFKGGKYMQAVIQYGKIVSWLEMEYGLSEKESKASES  314
             ESWEM+T EKLEQ+ IVKEKGT YFK GKY QA++QY +IVSWLE E  +   + + +++
Dr52    252  ESWEMNTIEKLEQSVIVKEKGTQYFKEGKYKQAIVQYKRIVSWLEHESSMQPDDEEKAKA  311

h51     315  FLLAAFLNLAMCYLKLREYTKAVECCDKALGLDSANEKGLYRRGEAQLLMNEFESAKGDF  374
               LAA+LNLAMCYLKL++   A+E CDKAL LD+ NEK L+RRGEA ++M EF+ AK DF
Dr52    312  LRLAAYLNLAMCYLKLQDANPALENCDKALELDANNEKALFRRGEALVVMKEFDMAKVDF  371

h51     375  EKVLEVNPQNKAARLQISMCQKKAKEHNERDRRIYANMFKKFAEQDAKE  423
             ++V+E+ P NKAA+ QIS+CQK  +E +E+D+R+YANMF+KFAE+DAKE
Dr52    372  QRVIELYPANKAAKSQISICQKHMREQHEKDKRLYANMFQKFAERDAKE  420
```

**Figure 4.2: FKBP51 and DrFKBP52 BLAST Sequence Alignment**

Sequence was aligned using BLAST alignment software. Accession numbers: NP_001139247 (FKBP51) and NP_958877 (DrFKBP52).

```
FKBP51          MTTDEGAKNNEESPTATVAEQGEDITSKKDRGVLKIVKRVGNGEETPMIGDKVYVHYKGK 60
DrFKBP52        MTAEEVVNEG-----CSIPIEGEDITPKKDGGVLKLVKKEGTGTELPMIGDKVFVHYVGT 55
                **::* .::.     .::. :*****.*** ****:**: *.* * *******:*** *.

FKBP51          LSNGKKFDSSHDRNEPFVFSLGKGQVIKAWDIGVATMKKGEICHLLCKPEYAYGSAGSLP 120
DrFKBP52        LLDGSQFDSSRDRGEKFSFELGKGQVIKAWDIGVATMKIGEICQLTCKPEYAYGAAGSPP 115
                * :*.:****:**.* * *.****************** ****:* ********:*** *

FKBP51          KIPSNATLFFEIELLDFKGEDLFED--GGIIRRTKRKGEGYSNPNEGATVEIHLEGRCGG 178
DrFKBP52        KIPPNATLLFQVELFDFRGEDITDDEDGGITRRIITKGEGYTKPNEGATVEVWLEGSHED 175
                ***.****:*::**:**:***: :*  *** **   *****::********: ***   .

FKBP51          RMFDCRDVAFTVGEGEDHDIPIGIDKALEKMQREEQCILYLGPRYGFGEAGKPKFGIEPN 238
DrFKBP52        RVFDERELKFEVGDGENLGLPLGVEKALQAMEQGEEALFTIKPKYGFGTAGSEKYNIPPN 235
                *:** *:: * **:**: .:*:*::***: *:: *:.:: : *:**** **. *:.* **

FKBP51          AELIYEVTLKSFEKAKESWEMDTKEKLEQAAIVKEKGTVYFKGGKYMQAVIQYGKIVSWL 298
DrFKBP52        ATLQYKIKMKAFEKAKESWEMNTIEKLEQSVIVKEKGTQYFKEGKYKQAIVQYKRIVSWL 295
                * * *::.:*:**********:* *****:.******* *** *** **::** :*****

FKBP51          EMEYGLSEKESKASESFLLAAFLNLAMCYLKLREYTKAVECCDKALGLDSANEKGLYRRG 358
DrFKBP52        EHESSMQPDDEEKAKALRLAAYLNLAMCYLKLQDANPALENCDKALELDANNEKALFRRG 355
                * * .:. .:.: :::: ***:**********:: . *:* ***** **: ***.*:***

FKBP51          EAQLLMNEFESAKGDFEKVLEVNPQNKAARLQISMCQKKAKEHNERDRRIYANMFKKFAE 418
DrFKBP52        EALVVMKEFDMAKVDFQRVIELYPANKAAKSQISICQKHMREQHEKDKRLYANMFQKFAE 415
                ** ::*:**: ** **::*:*: * ****: ***:***: :*::*:*:*:*****:****

FKBP51          QDAKEEANKAMGKKTSEGVTNEKGTDSQAMEEEKPEGHV 457
DrFKBP52        RDAKEADQEKEQDKKQNGSAME--IDENAAQEQTAA--- 449
                :****  ::   .*..:* : *   *.:* :*:..
```

**Figure 4.3: ClustalW2 Sequence Alignment of FKBP51 and DrFKBP52**

Sequence was aligned using ClustalW2 alignment software. Accession numbers: NP_001139247 (FKBP51) and NP_958877 (DrFKBP52).

### 4.2.2 DrFKBP52 and DrFKBP52-A111V Modeling

I-TASSER predicted five 3D models for each structural query. The loop region was stacked to determine whether the loop region maintains a similar conformation in all predictive models. While slight variations are seen they are insignificant, indicating that homology modeling for each model has a similar conformation. This gives us confidence that the predictive modeling for both targets, DrFKBP52 and DrFKBP52-A111V, were successful and precise. Because the A111V mutation is located at the proline-rich loop region, only the loop was isolated for further analysis, Figures 4.4 and 4.5.

**Figure 4.4: Overlapping of Wt-DrFKBP52 Homology Models**

Predictive models were overlapped using PyMOL, each model is highlighted in different colors.

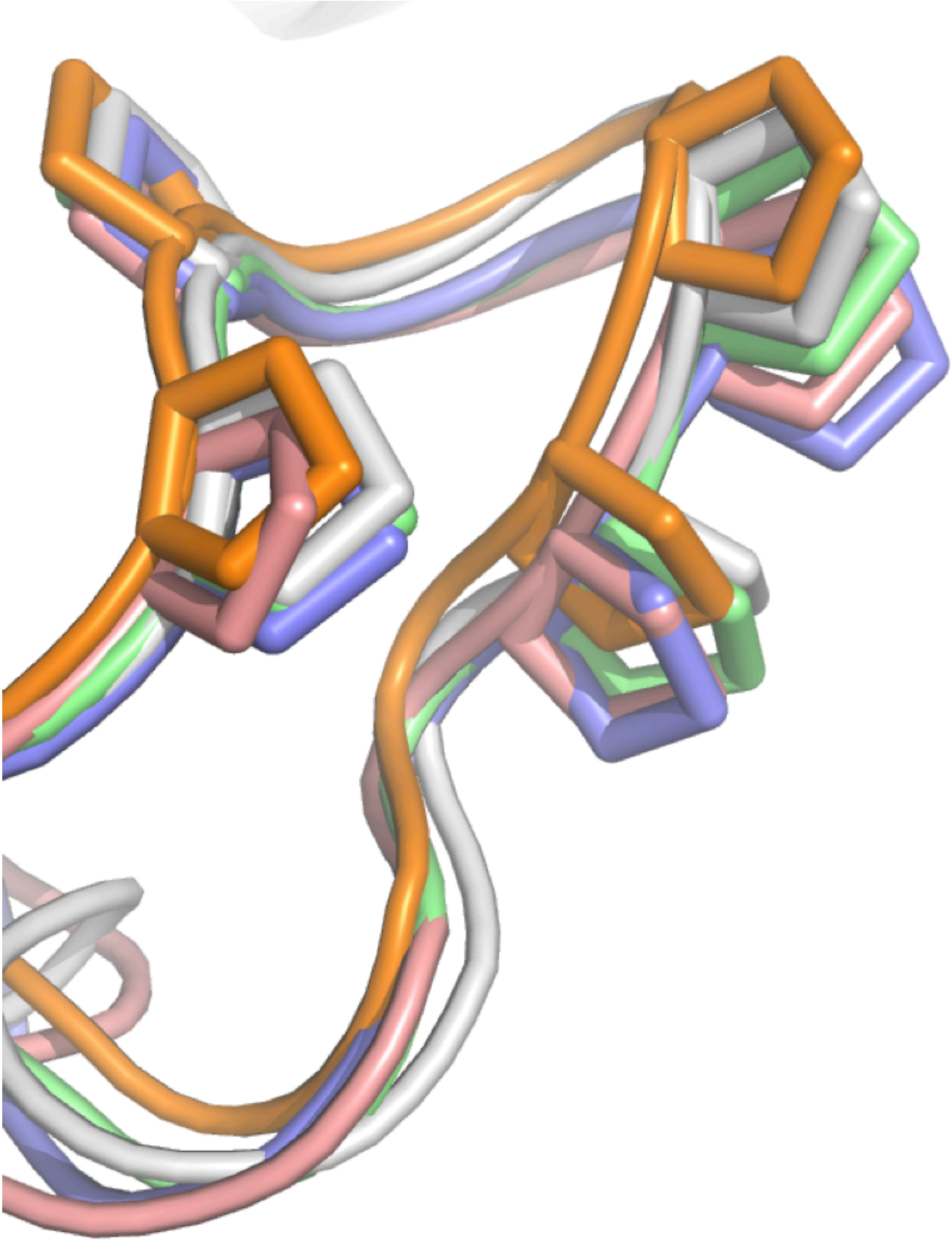


**Figure 4.5: Overlapping of DrFKBP52-A111V Homology Models**

Predictive models were overlapped using PyMOL, each model is highlighted in different colors.

### 4.2.3 Hydrophobicity Scale of Wt-DrFKBP52 and DrFKBP52-A111V

The hydrophobicity on the surface of the loop was assessed in wt-DFKBP52 and DrFKBP52-A111V to allow us to further evaluate what changes may be relevant in their functional divergence. We determined that there is no change in the overall shape or geometry of the loop, figure 4.4 and 4.5. However, the valine substitution at position 111 does affect the surface charge (to more neutral) and the hydrophobicity (to more hydrophobic) in the vicinity. This result is expected in all the predicted models, as the position of the Ala 111 or Val 111 relatively stays the same. Hydrophobicity models indicate an obvious change in surface area above the loop region between both FKBPs, see figure 4.6 A, B. This change in surface charge could be very important in understanding the necessary interaction between AR:hFKBP52.

A.

B.

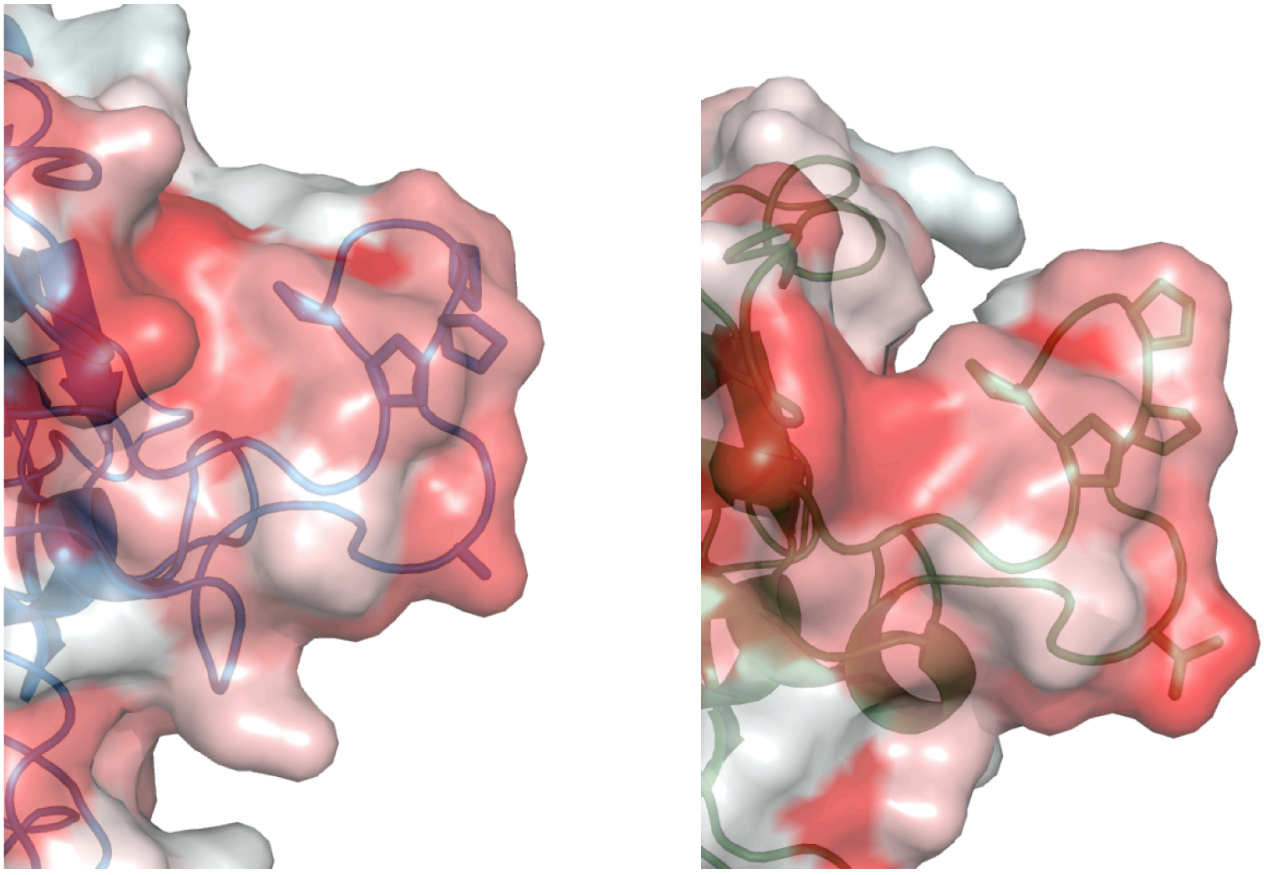


**Figure 4.6: Hydrophobicity Scale of DrFKBP52 and DrFKBP52-A111V**

The surface of the proline loop region was analyzed for hydrophobic intensity using PyMOL. (A) Wt-FKBP52. (B) DrFKBP52-A111V.

### 4.2.4 Comparison of FKBP Proline-Rich Loops

Homology modeling was utilized to further delineate the structural significance of position A111 and A116 in DrFKBP52 and hFKBP52 respectively. The bioinformatics software used compares one known protein structure to a highly similar unknown protein to predict the unknown protein structure. Using this method we discovered a very interesting conformational distinction between the DrFKBP52, hFKBP51 and hFKBP52 proline-loop regions. Figure 4.7 illustrates the predicted surface of the proline-rich loop from the various FKBP proteins and suggests a closed conformation for both hFKBP51 and DrFKBP52, while hFKBP52 has a more open conformation. The same is seen in the stick model, hFKBP51 L119 and S124 are in close association above the loop region and analogous residues in DrFKBP52 (P114 and P119) are also hovering over the loop region (Figure 4.7B). However, the DrFKBP52-A111V mutant is predicted to have a more open conformation similar to that of hFKBP52, both space-filled and stick model shows an unobstructed pocket above the proline-loop (Figure 4.7A, B). This is further corroborated by the hydrophobic surface modeling, which demonstrates how the proline-rich loop on both hFKBP52 and DrFKBP52-A111V, but not DrFKBP52 and hFKBP51, forms a hydrophobic pocket more conducible to protein-protein interaction (Figure 4.7C).

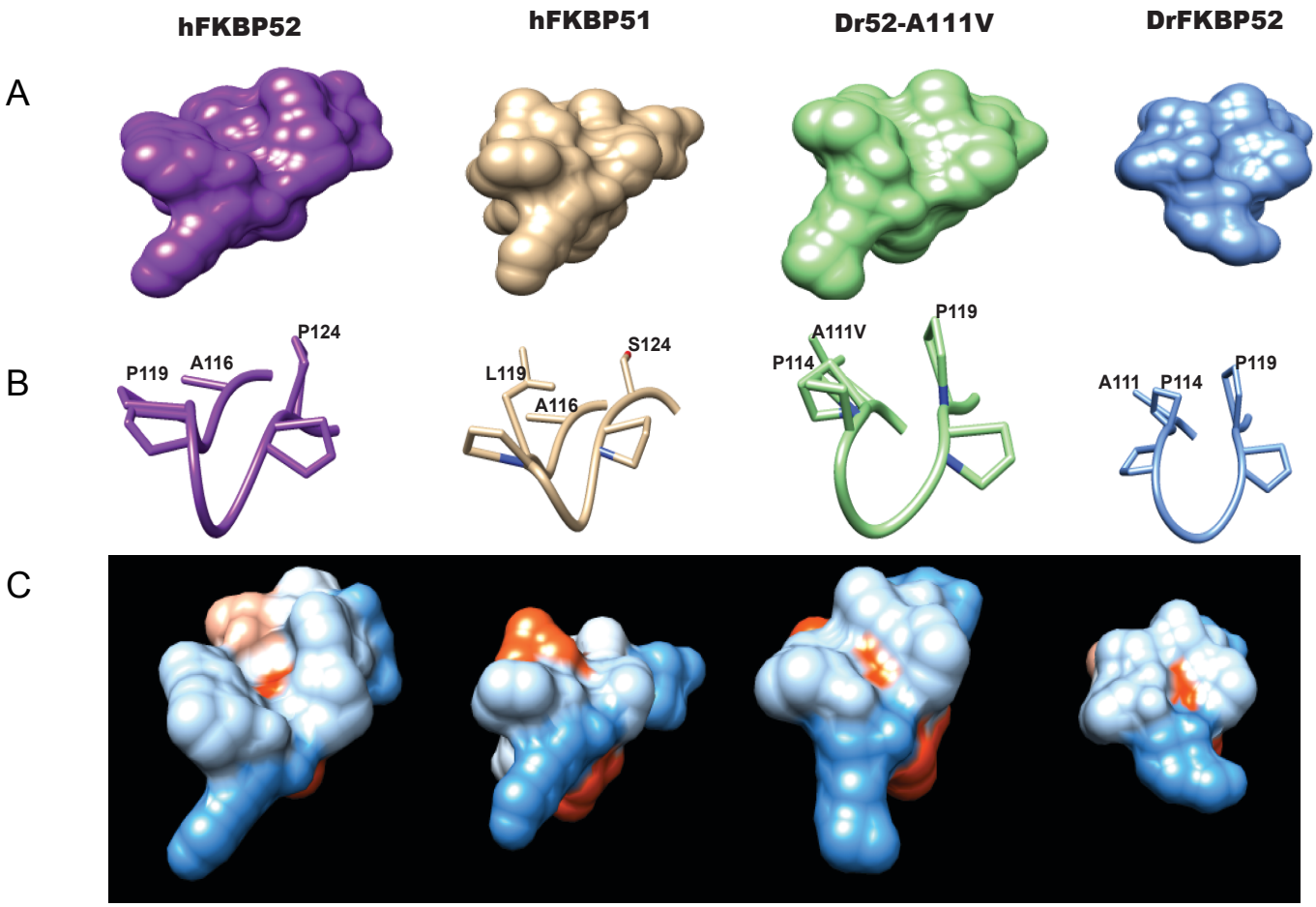



**Figure 4.7: Homology Modeling of the FKBP51, hFKBP52, DrFKBP52 and DrFKBP52-A111V Proline-Rich Loop Region**

(A) Homology modeling was used for comparison of hFKBP52 and FKBP51 to determine conformational changes induced by the DrFKBP52 A111V mutation. Crystal structures of FKBP51 (lkt0A.pdb), FKBP52 (1q1cA.pdb), DrFKBP52, and DrFKBP52-A111V are aligned; the respective FK1 domains were isolated and space-filled modeling and (C) hydrophobic surface conservation was done (B). Note that the only differences between FKBP51 and FKBP52 within the loop region are at positions 119 and 124.

## 4.3 DISSCUSION

To gain some insight into the potential role that the valine plays at position 111 and 116, predictive homology modeling was performed on DrFKBP52 and DrFKBP52-A111V. A close comparison of the corresponding loops in hFKBP52, FKBP51, DrFKBP52-A111V and DrFKBP52 (Figure 4.7A ,B, and C) reveals a striking structural difference due to the respective amino acids at position 111 and 116. The DrFKBP52 proline side chains (P114 and P119) protrude into a hydrophobic notch formed along the top of the loop, more similar to hFKBP51. The projection of these two residues is significantly altered by the addition of valine at position 111. The conformation of its proline spread more outwards, similarly to hFKBP52. Hydrophobic surface depictions show that hFKBP52, hFKBP51, DrFKBP52-A111V and DrFKBP52 all possess a hydrophobic surface above the proline-rich loop. This surface is blocked by the projection of surrounding residues in DrFKBP52 and FKBP51. Nevertheless, only hFKBP52 and DrFKBP52-A111V retain an open conformation compatible for protein-protein interaction. It is rational to conclude that DrFKBP52-A111V, in a manner analogous to hFKBP52, forms a functionally important contact via the FK1 hydrophobic notch with another component in the steroid hormone receptor complex. FKBP51 and DrFKBP52 lack this contact due to the altered loop conformation imposed by the leucine and proline, inhibiting adequate receptor interaction.

We propose that the FKBP52 FK1 domain, via the open conformation that exposes the hydrophobic notch formed by addition of valine at position 116, forms a specific contact with the receptor LBD and the highly positive region of the FK2 in the context of Hsp90 heterocomplexes and that this contact potentiates receptor response

to hormone. This theory is established in that we know that hFKBP52 binds Hsp90 directly and through this interaction potentiates the receptor. Additionally, studies have shown that FKBP52-dependent potentiation is localized to the receptor LBD (25, 13). Since hFKBP52 selectively potentiates the hormone-induced response of AR, GR, and PR, but not the activity of the mineralocorticoid and estrogen receptors, we conclude that the relevant FK1 interaction for potentiation is receptor specific, and not indirect through Hsp90 (109, 113). We suggest that the hydrophobic notch that forms above the proline-rich loop region could allow for a more efficient interaction, forming a LBD anchor. Through this study we conclude that prolines in the loop region are not the sole indicator of functionality, but structural integrity is key to maintain hFKBP52's potentiation of the steroid hormone receptor.

In summary we have shown that a single amino acid residue at position 111 and 116 near the PPIase loop plays a crucial role in the conformation integrity of the hydrophobic notch created by the proline-rich loop region, in both DrFKBP52 and hFKBP52. Since the PPIase loop is often involved in protein interactions, its accessibility is an important determining factor in the function of steroid receptor complexes. The identification of these critical residues is an important step toward understanding the mechanism of hFKBP52 interaction with steroid hormone receptors.

## REFERENCES


1. Society, A.C. *Cancer Statistics 2009 Presentation*. [website] 2009; Available from: http://www.cancer.org/docroot/PRO/content/PRO_1_1_Cancer_Statistics_2009_Present ation.asp.

2. Prostate Cancer Research Institute(PCRI).[website} 2002; Available from: http://www.prostate-cancer.org/education/andeprv/Myers_AndrogenResistance1.html

3. Prostate Cancer Research Institute(PCRI).[website} data unknown; Available from: http://www.prostate-cancer.org/education/preclin/pin.html

4. Lin, J.F., et al. (2007) *Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis.* Int J Cancer, 2007. 121(12): p. 2596-605.

5. Aghdasi B., et al. FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. Proc Natl Acad Sci U S A. 2001. 98(5): 2425–2430.

6. Riggs. D.L., Cox. M.,Tardif. H., Hessling. M, J., Buchner., Smith. D. (2007) Noncatalytic Role of the FKBP52 Peptidyl-Prolyl Isomerase Domain in the Regulation of Steroid Hormone Signaling. Mol and Cell Bio 27(24): 8658-8669.

7. Carrigan. P., Riggs. D., Chinkers. M., and Smith. D.(2005).Functional comparison of human and Drosophila Hop reveals novel role in steroid receptor maturation. JBC. 2005. 280 (10):8906-11.

8. M.Beato and J. Klug.(2000).Steroid hormone receptors: an update. Human Reproduction 6 (3):225-236.

9. Millipore. “Transcription Androgen Receptor nuclear signaling”.[website] 2009. Available from: http://www.millipore.com/pathways/pathviewer.do?searchTerms=AR+and+AKT&disease AreaIds=&cellProcessIds=&pathwayId=11

10. Pratt W.B and Toft D.O.(1997) Steroid Receptor Interaction with Heat Shock Protein and Immunophilin Chaperones. Endo Rev. 18(3):306-356.

11. Ward B.K., Mark PJ., Ingram D.M., Minchin R.F, and Ratajczak T.(1999) Expression of the estrogen receptor-associated immunophilins, cyclophilin 40 and FKBP52, in breast cancer. Breast Cancer Res Treat. 58(3):267-80.

12. Stéphane Terry , Xuezhen Yang , Min-Wei Chen , Francis Vacherot , and Ralph

Buttyan (2006). Multifaceted interaction between the androgen and Wnt signaling pathways and the Implication for prostate cancer.JCB. 99(2):402-10

13. Xu-Bao Shi., et al., (2002) Functional Analysis of 44 Mutant Androgen Receptors from Human Prostate Cancer. Cancer Res 62: p.1496-1502

14. Perpin E.E., (2007) A surface on the androgen receptor that allosterically regulates coactivator binding. PNSA 104(41): 16074-16079

15. Fisher, Jane S. (2004) Environmental anti-androgen and male reproductive health: Focus on phthalates and testicular dysgenesis syndrome. Reproductive 127: 305-315

16. Flynn J.C., Prapapanich V., et al. (2005) Physiological Role for the Cochaperone FKBP52 in Androgen Receptor Signaling. Mol Endo 19(6):1654-1666 .

17. Scott M. Dehm and Donald j. Tindall.(2007) Androgen Receptor structural and functional Elements: Role and regulation in Prostate Cancer. Molecular Endo 21(12):2855-2863

18. Quigley C. A., et al. (1995) Androgen Receptor Defects: Historical, Clinical, and Molecular Perspectives. *Endocr Rev.* 16: 271-321.

19. Claessens F., et al. (2008) Diverse roles of androgen receptor (AR) domains in AR- mediated signaling. Nucl Recept Signal. V 6:e008.

20. Saomarelli J. A., Lee S. Y., Herrera R. J. (2008) Structure-based classification of 45 FK506-binding proteins. Proteins 72(1):197-208.

21. Lu K.P., et al. (2007) Prolyl cis-trans isomerization as a molecular timer. Nat Chem Bio. 3(10) 619-629.

22. Schiene-Fischer C., Yu C. (2001) Receptor accessory folding helper enzymes: the functional role of peptidyl prolyl cis/trans isomerases. FEBS. 495:1-6.

23. Aghdasi B., et al. (2001) FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. Proc Natl Acad Sci 98(5): 2425–2430.

24. Cheung-Flynn J., et al. (2003) C-terminal Sequences outside the Tetratricopeptide Repeat Domain of FKBP51 and FKBP52 cause differential binding to Hsp90. Bio Chem, 278:19 p. 17394-17394.

25. Riggs D. L., PJ. Roberts, and SC. Chirillo, *et al. (2003)* The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. *EMBO J* 22: (5) 1158-1167

26. Davies T.H., et al. (2005) Differential control of glucocorticoid receptor hormone-binding function by tetratricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506. *Biochemistry*. 44: (6) 2030-2038.

27. Pirkl K.,Buchner J.(2001) Functional analysis of the Hsp90-associated human peptidyl prolyl cis/trans isomerases FKBP51, FKBP52 and Cyp40. *J Mol Biol* 308: (4) 795-806.

28. Scheufler C. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70 Hsp90 multichaperone machine. Cell 101(2):199-210.

29. Futer O. (1995) FK506 Binding Protein Mutational Analysis. Bio Chem 270:32 p. 18935-18940.

30. Cheung-Flynn J., et al. (2005) Physiological Role for the Cochaperone FKBP52 in Androgen Receptor Signaling. Mol Endo. 19(6): 1654-1666.

31. Sinars C. R., et al. (2003) Structure of the large FK506 binding protein FKBP51, anHsp90- Binding protein and a component of steroid receptor complexes. PNAS. 100:3 p 868-873

32. Pratt W.B and Toft D.O.(1997) Steroid Receptor Interaction with Heat Shock Protein and Immunophilin Chaperones. Endo Rev 18(3):306-356

33. Denny, W. B., D. L. Valentine, Reynolds P. D., Smith D. F. (2000).Squirrel Monkey Immunophilin FKBP51 Is a Potent Inhibitor of Glucocorticoid Receptor Binding. ENDO 141:4107-4113

34. Gross K.L., et al. (2008) Androgen Resistance in Squirrel Monkeys. Comparative Med. 58(4)381-388

35. Yong W., et al. (2007) Essential Role for co-chaperone FKBP52 but not FKBP51 in Androgen receptor- mediated Signaling and Physiology. Bio Chem. 282(7): 5026-36

36. Wochnik M.G., Ruegg .J., Abel. A., el at. (2005). FK506-binding Proteins 51 and 52 differentially regulate dynein Interaction and Nuclear translocation of Glucocorticoid Receptor in mammalian cells.JBC 280(6): 4609-4616

37. Chambraud .B., Belabes .H., Fontaine-Lenoir .V.(2007).The immunophilin FKBP52 specifically binds to tubulin and prevents microtubule formation. FASEB J 21:2787-2797.

38. Buchner J.(1999).Hsp90 & CO.-holding for folding. Elsevier Science 136-141.

39. SC. Nair, RA. Rimerman, and EJ. Toran, *et al.(1997)* Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. *Mol Cell Biol* 17: (2) 594-603.

40. Cox M. B., Smith D.F., et al. (2007) Functions of HSP90-Binding FKBP Immunophilins. Networking of Chaperone by Co-chaperones. Chapter 2:13-20.

41. Wu B., et al. (2004) 3D structure of human FK506-binding protein 52: Implications for the assembly of the GR/ Hsp90/immunophilin heteropcomplex. PNAS. 101(22): 8348-53.

42. Carrigan. P., Riggs. D., Chinkers. M., and Smith. D. (2005). Functional comparison of human and Drosophila Hop reveals novel role in steroid receptor maturation. JBC 280 (10):8906-11

43. Davies T.H., Sánchez E.R. (2005) FKBP52 .J Biochem Cell Biol 37(1):42-7. Review.

44. Matsumoto T. et al.(2013) The Androgen Receptor in Health and Disease. Annual Review of Physiology 75:201-224.

45. Werner R. et al. (2008) In-vitro characterization of androgen receptor mutations associated with complete androgen insensitivity syndrome reveals distinct functional deficits. Sex Dev 2(2) 73-83.

46. Kumar R. et al. (2011) Role of androgen receptor CAG repeat polymorphism in prostate cancer, and spinal and bulbar muscular atrophy. Life Science 88(13-14) 565-71.

47. Fischbeck K.H. (2012). Developing treatments for spinal and bulbar muscular atrophy. Prog Neurobiology 99(3):257-261.

48. Howlader N NA, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). (2009) SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations). (National Cancer Institute, Bethesda).

49. Passoni NM*, et al.* (2012). Clinical and diagnostic assessment for therapeutic decisions in prostate cancer. *Q J Nucl Med Mol Imaging* 56(4):321-330.

50. Edwards DP. (2000). The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. *J Mammary Gland Biol Neoplasia* 5(3):307-324 .

51. Claessens F*, et al.* (2008) Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. *Nucl Recept Signal* 6:e008.

52. Anonymous (2012) American Cancer Society: Cancer Facts and Figures (American Cancer Society, Atlanta).

53. Siegel R*, et al.* (2012) Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin* 62(4):220-241.

54. Heemers H.V, *et al.* (2004). Unraveling the complexities of Androgen Receptor Signaling in Prostate Cancer cells. Cancer Cell 15(4):245-247.

55. Nazareth L.V. (1996) Activation of the human androgen receptor through a protein kinase A signaling pathway. Journal Biol Chem 271(33). 199000-7.

56. Sadar M.D, *et al. (*1999*)* Prostate cancer: molecular biology of early progression to androgen independence. Endocrine-Related Cancer (6) 487-502.

57. Ueda T. *et al*. (2002) Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. Journal Biol Chem 277(9) 7076-85.

58. Yardy GW & Brewster SF (2005) Wnt signalling and prostate cancer. *Prostate Cancer Prostatic Dis* 8(2):119-126.

59. McCarty M.F., et al. (2005) Targeting multiple signaling pathways as a strategy for managing prostate cancer: multifocal signaling modulation therapy. Integrative Cancer Therapies 3(4): 349-80.

60. Sarker D.,et al.(2009).Targeting the PI3K/AKT Pathway for the Treatment of Prostate Cancer. Clinical Cancer Research (10)1078-0432.

61. Jiang Z.K., *et al*. (2013). Rapamycin Enhances Adenovirus-Mediated Cancer Imaging and Therapy in Pre-Immunized Murine Hosts. PLoS ONE 8(9): e73650. doi:10.1371/journal.pone.0073650

62. Lee G. T. (2014). Prostate cancer bone metastases acquire resistance to androgen deprivation via WNT5A-mediated BMP-6 induction. British Journal of Cancer 110: 1634-1644. doi:10.1038/bjc.2014.23

63. Hay CW, McEwan IJ. (2012). The Impact of Point Mutations in the Human Androgen Receptor: Classification of Mutations on the Basis of Transcriptional. PLoS ONE 7(3): e32514. doi:10.1371/journal.pone.0032514

64. Tilley W. D., *et al* .(1996). Targeting the androgen receptor: improving outcomes for castration- resistant prostate cancer. Endocrine-Related Cancer 11 459–47

65. Buchanan G., et al. (2001). Collocation of Androgen Receptor Gene Mutations in

Prostate Cancer. Clinical Cancer Research 7:1273.

66. Shiota M., et al (2011). Increased androgen receptor transcription: a cause of castration-resistant prostate cancer and a possible therapeutic target. Journal Mol Endocrinol 11:0018

67. Yardy GW & Brewster SF (2005) Wnt signalling and prostate cancer. *Prostate Cancer Prostatic Dis* 8(2):119-126.

68. Tilley W.D., et al. (1990). Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell lines. Journal of Biological Chemistry 265(23):13776-81.

69. Gregory C. W., et al. (1998). Androgen receptor expression in androgen-independent prostate cancer is associated with increase expression of androgen-regulated genes. Cancer Research 58:5218-5724.

70. Zegarra-Moro O.L., et al. (2002). Disruption of Androgen Receptor Function Inhibits Proliferation of Androgen-refractory Prostate Cancer Cells. Cancer Research 62:1008-1013

71. Scher H. I., et al. (2004). Targeting the androgen receptor improving outcomes for castration resistant prostate cancer. Endocrine-Related Cancer 11: 459-476.

72. Scher H. I., et al. (2005). Biology of progressive, castration-resistant prostate cancer: Directed therapies targeting the androgen-receptor signaling axis. JCO 2005:8253-8261.

73. Friedlander T.W., et al. (2011). Common Structural and Epigenetic Changes in the Genome of Castration Resistant Prostate Cancer. Journal Cancer Research 10.1158/0008-5472.

74. Courtney KD., et al. (2012). The evolving paradigm of second-line hormonal therapy options for castration-resistant prostate cancer. Curr Opin Oncol 24(3): 272-7.

75. Chang K. H., et al. (2013). A gain-of-function mutation in DHT synthesis in castration-resistant prostate cancer. Cell Press 154(5): 1074-84**.**

76. Vasaitis T. S., et al. (2011). CYP17 inhibitors for prostate cancer therapy. J Steroid Biochem Mol Biol 125(1-2): 23-31.

77. Miller W. L. (2011). The syndrome of 17,20 lyase deficiency. Journal of Clin Endo and Meta 97(1):59-67.

78. Kim W., et al. (2012). Androgen receptor directed therapies in castration-resistant

metastatic prostate cancer. Current Treatment Option in Oncology 13(2):189-200.

79. Mariano A. E., et al. (2013). Highly Potent and Selective Nonsteroidal Dual Inhibitors of CYP17/CYP11B2 for the Treatment of Prostate Cancer To Reduce Risks of Cardiovascular Diseases. *Journal of Medicinal Chemistry 56* (15), 6101-6107.

80. Chen. Y., et al. (2009). Antiandrogens and androgen depleting therapies in prostate cancer: novel agents for an established target. Lancet Oncol 10(10): 981-991.

81. Chen C.D., et al. (2004). Molecular Determinants of Resistance to Antiandrogen Therapy. Nature Medicine 10(1).

82. Trepel J, Mollapour M, Giaccone G, & Neckers L (2010) Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer* 10(8):537-549.

83. Mostaghel E.A. (2014). Abiraterone in the treatment of metastatic castration-resistant prostate cancer. Cancer Manag Reearch 6:39-51.

84. Korpal M. (2013). An F876L Mutation in Androgen Receptor Confers Genetic and Phenotypic Resistance to MDV3100 (Enzalutamide). Cancer Discovery 3:1039.

85. Carver B.S. et al. (2011). Reciprocal Feedback Regulation of PI3K and Androgen Receptor Signaling in PTEN-Deficient Prostate Cancer. Cancer Cell 19(5) 575-586.

86. Rohl A., et al. (2013). The chaperone Hsp90: changing partners for demanding clients. Trends in Biochemical Sciences. 38(5):253-262.

87. Trepel J., et al. (2010). Targeting the Dynamic HSP90 Complex in Cancer. 10(8):537-549.

88. Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, & Tindall DJ (2008) Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res* 68(13):5469-5477.

89. Wilson CM & McPhaul MJ (1994) A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc Natl Acad Sci U S A* 91(4):1234-1238.

90. Hu R, Isaacs WB, & Luo J (2011) A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. *Prostate* 71(15):1656-1667.

91. Lavery DN & McEwan IJ (2005) Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. *Biochem J* 391(Pt 3):449-464.

92. Khorasanizadeh S & Rastinejad F (2001) Nuclear-receptor interactions on DNA-response elements. *Trends Biochem Sci* 26(6):384-390 .

93. Dehm SM & Tindall DJ (2007) Androgen receptor structural and functional elements: role and regulation in prostate cancer. *Mol Endocrinol* 21(12):2855-2863.

94. Quigley C.A., et al. (1995). Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16(3):271-321.

95. Myung J. K., et al. (2013). An androgen receptor N-terminal domain antagonist for treating prostate cancer. Journal Clin Invest 123(7): 2948-2960.

96. Goto K., et al. (2003). Activation function-1 domain of androgen receptor contributes to the interaction between two distinct subnuclear compartments. Jou of Stero Biochem and Mol Biol 85:201-208.

97. Lavery D. N., et al. (2011). Androgen Receptor Signalling in Prostate Cancer: The Functional Consequences of Acetylation. Jou Biomed Biotech 2011:7.

98. Poukka H., et al. (2000). Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). 97(26):14145-50.

99. Steensel B. V., et al. (1995). Domains of the human androgen receptor and glucocorticoid receptor involved in binding to the nuclear matrix. Journal of Cell Biochem 57(3):465-478.

100. Beato M & Klug J (2000) Steroid hormone receptors: an update. *Hum Reprod Update* 6(3):225-236.

101. Schoenmakers E., et al. (1999). Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Journal of Biochemical 34(515-521).

102. Coffey K., et al. (2012). Characterization of a Tip60 specific inhibitor, NU9056, in prostate cancer. PLoS One 7(10).

103. He B*, et al.* (2006) Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. *J Biol Chem* 281(10):6648-6663.

104. Weatherman RV, Fletterick RJ, & Scanlan TS (1999) Nuclear-receptor ligands and ligand-binding domains. *Annu Rev Biochem* 68:559-581.

105. Estebanez-Perpina E*, et al.* (2005) The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. *J Biol Chem* 280(9):8060-8068.

106. Edwards DP (2000) The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. *J Mammary Gland Biol Neoplasia* 5(3):307-324.

107. Echeverria PC*, et al.* (2009). Nuclear import of the glucocorticoid receptor-hsp90 complex through the nuclear pore complex is mediated by its interaction with Nup62 and importin beta. *Mol Cell Biol* 29(17):4788-4797.

108. Cox MB, and Smith, D.F. (2006) Functions of the Hsp90-Binding FKBP Immunophilins. *The Networking of Chaperones by Cochaperones*, ed Blatch GL (Eurekah.com).

109. Barent RL*, et al.* (1998) Analysis of FKBP51/FKBP52 chimeras and mutants for Hsp90 binding and association with progesterone receptor complexes. *Mol Endocrinol* 12(3):342-354.

110. De Leon JT*, et al.* (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(29):11878-11883 .

111. Cox MB*, et al.* (2007) FK506-binding protein 52 phosphorylation: a potential mechanism for regulating steroid hormone receptor activity. *Mol Endocrinol* 21(12):2956-2967.

112. Bracher A., et al. (2013) Crystal structure of fee and ligand-bound FK1 and FK2 domain segment of FKBP52 reveal a flexible inter-domain hinge. J Mol Bio 425(22): 4134-4144.

113. Sliverstein A. M., et al. (1999) Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. J Biol Chem 274(52):36980-6.

114. Silva H. B., et al. (2013) Dissecting Major Signaling Pathways throughout the Development of Prostate Cancer. Prostate cancer. 2013: 930612 .

115. Tranguch S*, et al.* (2005) Cochaperone immunophilin FKBP52 is critical to uterine receptivity for embryo implantation. *Proc Natl Acad Sci U S A* 102(40):14326-14331.

116. Krieger,E., Nabuurs,S.B. and Vriend,G. (2003) Homology modeling. Methods Biochem. Anal., 44, 509–523.

## CURRICULUM VITA

Diondra Harris earned her Bachelor of Science degree in biology from Stillman College in Tuscaloosa, AL in 2004. She attended The University of Alabama at Huntsville to study nursing from 2006 to 2007. In Fall 2007, she joined the doctoral program at the Department of Biological Sciences at UTEP to study the molecular biology of prostate cancer.

Dr. Harris has been the recipient of numerous honors and awards at UTEP. She received the Alliance for Graduate Education and the Professoriate (AGEP) and the Research Initiatives for Scientific Enhancement award. Additionally, she received a UTEP Graduate School Research Award. While pursuing her degree, Dr. Harris worked as a teaching assistant of Immunology, Anatomy and Physiology and General Biology, and she worked as a research assistant under Dr. Marc Cox. She was also a biology professor at franklin community college, where she educated pre-nursing and pre-dental students basic biological concepts.

Dr. Harris has presented her research at numerous conferences, including a poster at SACNAS in El Paso, TX, an oral presentation at the UTEP Graduate Research Exposition in El Paso, a poster presentation at the Emory University Research Symposium in Atlanta, GA. She also was award 2$^{nd}$ place for her research poster at Emory research symposium.

*Dr. Harris's publications and co-authorships have been featured in Journal of Biological Chemistry, Current Protein and Peptide Science. Her final publication is in preparation and should be released in Fall 2014.*

Her dissertation project entitled "A Comparative Approach to Assessing the Functional and Structural Characteristics of Human FKBP52 in the Regulation of Steroid Hormone Receptor Signaling Pathway", was supervised by Dr. Marc B. Cox. Upon graduation, Dr. Harris plans to cultivate her research skills as a post-doctoral fellow in cancer genetics and pharmacogenetics/genomics, in hopes of becoming an accomplished research scientist.

Permanent Address:
2223 Apache Drive NW
Huntsville, AL 25810

This dissertation was typed by Diondra C. Harris