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# A NOVEL FKBP52/β-CATENIN COMPLEX SPECIFICALLY REGULATES ANDROGEN RECEPTOR ACTIVITY

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2013

## Dedication

This project is and has always been dedicated to my Lord and Savior Jesus Christ. I have always been fascinated by your handiwork. Thank you for allowing me to discover the intricacies of just a tiny piece of your design. I pray that you will use this work to extend life or improve its quality for prostate cancer patients worldwide. Thank you for giving me this opportunity to learn and to grow in my appreciation of your design. All glory and praise to you!

# A NOVEL FKBP52/β-CATENIN COMPLEX SPECIFICALLY REGULATES ANDROGEN RECEPTOR ACTIVITY

by

CHERYL LYNNE STORER, B.S.

DISSERTATION

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- Dr. Fletterick's laboratory from the University of California San Francisco
- Dr. Anders Strom from the University of Houston
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## Abstract

The androgen receptor complex plays an essential role in prostate cancer progression due to exploitation of the androgen receptor (AR) as a transcription factor. The final stage of the receptor complex consists of a dimerized receptor, a dimeric heat shock protein (Hsp90), the cochaperone p23, and an immunophilin. Hormone-dependent prostate cancer progresses due to key interactions between the androgen receptor complex and its ligand 5α-dihydrotestosterone (DHT). While current treatments focus on blocking the androgen receptor-ligand interactions, these therapies are no longer effective in advanced stage, hormone-refractory prostate cancer (HRPC). Therefore, we have been interested in targeting other members of the androgen receptor complex and signaling cascade, namely the immunophilin FK506 Binding Protein 52 (FKBP52) and the cell signaling protein β-catenin. FKBP52 has the ability to potentiate activity of the androgen, progesterone, and glucocorticoid receptors and is a novel prostate cancer biomarker. β-catenin is a member of the cellular adhesion complex with E-cadherin in healthy cells, but in aberrant situations, β-catenin actively promotes cancer progression and invasion.

Our data in *FKBP52* knockout mouse embryonic fibroblasts (52 KO MEF) and *FKBP52* Knock down 22RV1 cell lines suggests that FKBP52 and β-catenin work in tandem to synergize AR transcriptional activity. When AR, FKBP52, and β-catenin are cotransfected, AR signaling synergizes under levels of very low DHT, as tracked by luciferase reporter assays. The synergy is so great that it dwarfs the potentiation of FKP52 and regulation of β-catenin alone. According to pull down assays, FKBP52 and β-catenin can bind in the absence of other proteins. Luciferase assays of mutant FKBP52 proteins suggest that FKBP52's binding to β-catenin involves the FKBP52 proline-rich loop but acts independently of Hsp90 binding. Interestingly, it appears that this synergistic effect is DNA sequence-specific, suggesting that the regulation by these proteins

occurs at the transcriptional level. We have successfully abrogated the synergism of these proteins using a small molecule inhibitor compound MJC13, developed in our laboratory. According to surface plasmon resonance studies, this molecule binds the androgen receptor at the Binding Function 3 (BF-3) regulatory surface, the putative interaction surface for FKBP52 regulation of receptor. MJC13 potency is not improved with the combined administration of the anti-androgen Bicalutamide or the Hsp90 inhibitors KU174 or 17AAG. Studies in 22RV1 prostate cancer cells comparing control cells to FKBP52 knock down cells reveal the obligatory role of FKBP52 in β-catenin potentiation of AR. In the absence of FKBP52, β-catenin transfection barely increases AR signaling over basal levels, but when FKBP52 is present, β-catenin is a strong coactivator of transcription by AR. This information will lead to the development of new prostate cancer therapies.

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## 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

AD2 – autonomous activation domain 2

ADT– androgen deprivation therapy

AF-2– Activation Function 2

AR – androgen receptor

AHA-1 – activator of Hsp90 ATPase1

APC – adenomatous polyposis coli

ARA70 – AR-associated protein 70

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

BRG-1 – Brahma related gene 1

CARM1 – coactivator-associated arginine methyltransferase

CBF – CCAAT binding factor

CDC37 – cell division cycle 37 homologue

CK 5, 8, and 18 – cytokeratin 5, 8, and 18

ChIP – chromatin immunoprecipitation

CHIP – COOH terminus of the Hsp70-interacting protein

Co-IP – coimmunprecipitation

COX-2 – cyclooxygenase 2

CRPC – castration-resistant prostate cancer

CyP – cyclophilin

DBD – DNA Binding Domain

DHT – 5α-dihydrotestosterone

DM – double mutant

DMEM – Dulbecco's modified eagle medium

DMSO – dimethyl sulfoxide

DOC- deoxycorticosterone

$EC_{50}$ – 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

FKBP51– FK506 Binding Protein 51

FKBP52 – FK506 Binding Protein 52

FSH – follicle stimulating hormone

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GR – glucocorticoid receptor

GRIP1 – glucocorticoid receptor interacting protein 1

GSK3 β – glycogen synthase kinase 3 β

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

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

MMP-7 – matrix metalloprotease 7

MPER – mammalian protein extraction reagent

MR – mineralocorticoid receptor

mTOR – mammalian target of rapamycin

NCI – National Cancer Institute

NR – nuclear receptor

Nup62 – nucleoporin 62

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- γ – peroxisome proliferator activated receptor γ

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

RSC – chromatin structure remodeling

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

SCID – severe combined immunodeficiency

SMYD-2 and 3 – SET and MYND domain containing 2 and 3

SRC2 – steroid receptor coactivator 2

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

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 A BASIC MODEL OF ANDROGEN RECEPTOR SIGNALING PATHWAYS

The androgen receptor (AR) is a member of the Type I steroid hormone receptor (SHR) family, also comprised of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and estrogen receptor (ER) (1). AR is a 110 kDa protein which is transcribed from a gene located on the X chromosome (2). Because of its importance in the regulation of androgen-mediated genes, AR localization is tightly regulated. The androgen receptor, like other SHRs, does not exist as a lone protein in the cytoplasm; it is always found in conjunction with other proteins, which regulate both its conformational state and localization. The proteins of the androgen receptor complex associate with the receptor in the cytoplasm to maintain the receptor in an appropriate conformation to bind ligand as well as to localize the receptor in the cytoplasm until ligand binding occurs. The classic model maintains that the receptor dissociates from the complex upon hormone binding and travels to the nucleus. However, the discovery that the entire mature MR complex of proteins, including heat shock protein 90 (Hsp90), p23, MR, and FK506 Binding Protein 52 (FKBP52), is capable of traveling to the nucleus (3) certainly arouses the possibility that the AR complex could translocate as well. Hsp90-bound steroid hormone receptors are incapable of binding DNA (4). Therefore, it is safe to say that while the location of detachment from the Hsp90 complex is still being determined, it is dissociated from the complex when it binds DNA.

Androgens, the ligands for AR, are signaling molecules generated in the adrenal cortex, ovaries, and testes through transformations of cholesterol. Bound by carrier molecules, androgens travel to target tissues in the body through the bloodstream (5). Once androgens contact target tissues, they travel by diffusion into the cell. It is difficult to estimate the amount of testosterone that actually reaches target tissues *in vivo*, since the bioavailability of the

hormone is regulated by sex hormone binding globulin (SHBG). While androgen levels (such as 5α-dihydrotestosterone (DHT) and androstanediol glucuronide (AAG)) can be quantified from serum samples, it is difficult to generalize about "normal" levels of androgens due to interpersonal variability in clearance rates and bioavailability. Increased levels of testosterone or decreased levels of SHBG are associated with prostate cancer (6).

The conformation of the steroid hormone receptor must be primed for hormone binding by a series of events involving a number of chaperones and cochaperones, as seen in **Figure 1.1.** This figure is primarily based upon studies of GR and ER (4, 7). As soon as the receptor leaves the ribosome, the receptor complexes with chaperones such as the heat shock proteins (8). The early complex is characterized by the association of Hsp40 and Hsp70 with the receptor. Hsp40 initiates Hsp70's ATPase activity, activating Hsp70 to chaperone the receptor into an appropriately folded state. In the intermediate complex, the receptor binds the adaptors Hsp Interacting Protein (HIP) and Hsp Organizing Protein (HOP) or the chaperone cofactors BCL-2 athanogene-1 (BAG-1) and COOH terminus of the Hsp70-interacting protein (CHIP) (7). If the receptor has not folded properly, BAG-1 transfers the receptor complex to CHIP, a ubiquitin ligase which tags the protein for the proteasomal degradation pathway (9). However, if the receptor is in an appropriate conformation, Hsp40 is displaced with HIP, a protein that increases the stability of the ADP-bound form of Hsp70. HOP also binds and promotes the transfer of the receptor from Hsp70 to the Hsp90 dimer.

The mature complex is characterized by the association of the receptor with the Hsp90 dimer, the cochaperone p23 which stabilizes the Hsp90 interaction with the receptor by keeping it in an ATP-bound state, and an immunophilin (4). Immunophilins are proteins which bind immunosuppressive drugs but may or may not participate in immunosuppression when bound to

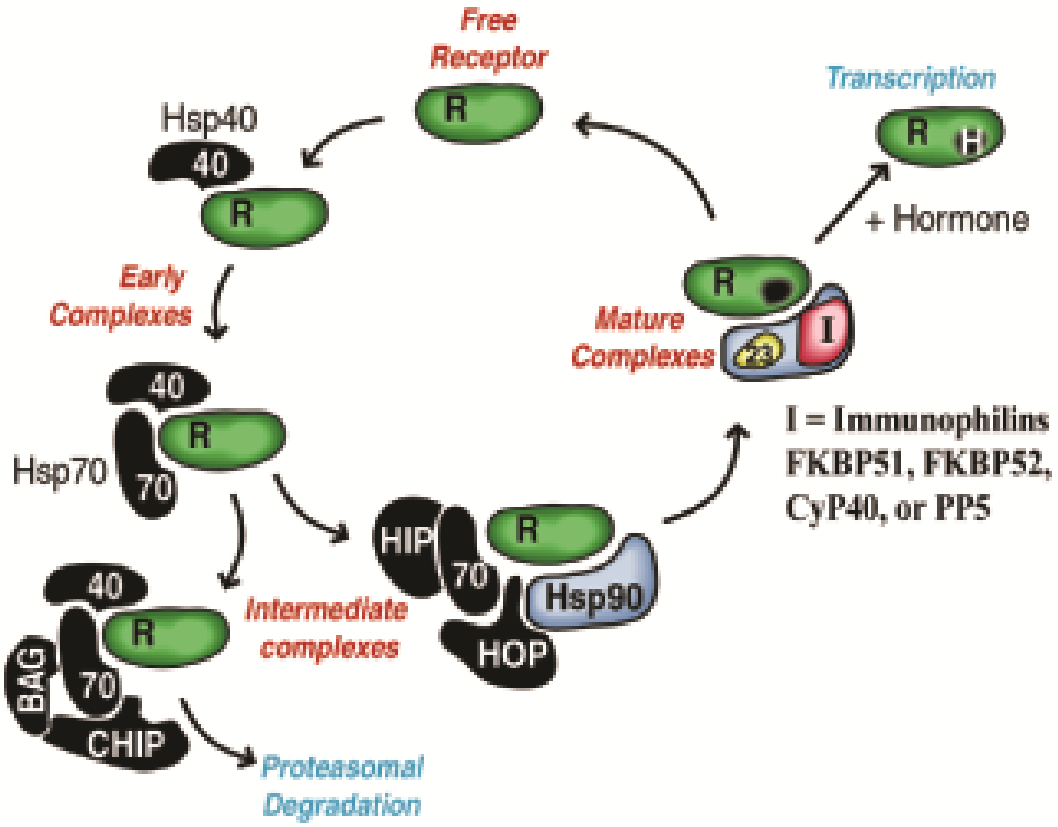



Figure 1.1: Steroid Hormone Receptor Maturation Cycle

Figure 1.1 depicts the association of the steroid hormone receptor with chaperones and cochaperones in the early, intermediate, and mature steroid hormone receptor complexes.

those drugs. Immunophilins which associate with the steroid hormone receptors to either increase or decrease their activity as transcription factors are FKBP51, FKBP52, Cyclophilin 40 (Cyp40), or serine/threonine protein phosphatase 5 (PP5) (4).

Formation of the mature complex is required to prime the receptor for high-affinity binding of hormone (10). The immunophilins FKBP51 and FKBP52 may bind AR at this stage of the complex. FKBP52 is associated with potentiation of AR (11), GR (12), and PR (13) signaling in response to hormone, whereas FKBP51 normally antagonizes steroid receptor signaling (10).

**Figure 1.2** presents a summary of the receptor's interactions with other factors in the non-cancerous cell. When hormone enters the cell, it binds the mature complex of the receptor, presumably in the cytoplasm. While Hsp90 is indeed required for the initial binding of the receptor to hormone, it is not required for transcriptional events in the nucleus. Following hormone binding, the receptor homodimerizes by attaching DNA Binding Domain (DBD) to DBD, Ligand Binding Domain (LBD) to LBD, and N-terminus to C-terminus. The receptor then binds hormone response elements within the DNA at the conserved consensus site shared by AR, PR, GR, and MR. Later the receptor returns to the cytoplasm where it enters the receptor maturation cycle again, destined for either successive hormone binding or degradation (2).

## 1.2 Androgen Receptor Isoforms and Structure

The androgen receptor exists in multiple isoforms in normal tissues. Androgen receptor isoforms A and B are the most prevalent forms found in normal human tissues and are generated by alternative splicing (14). AR-A is an 87kDa, truncated form of the receptor, whereas AR-B is a

110 kDa protein which contains 23 additional amino acids at the N-terminal domain.

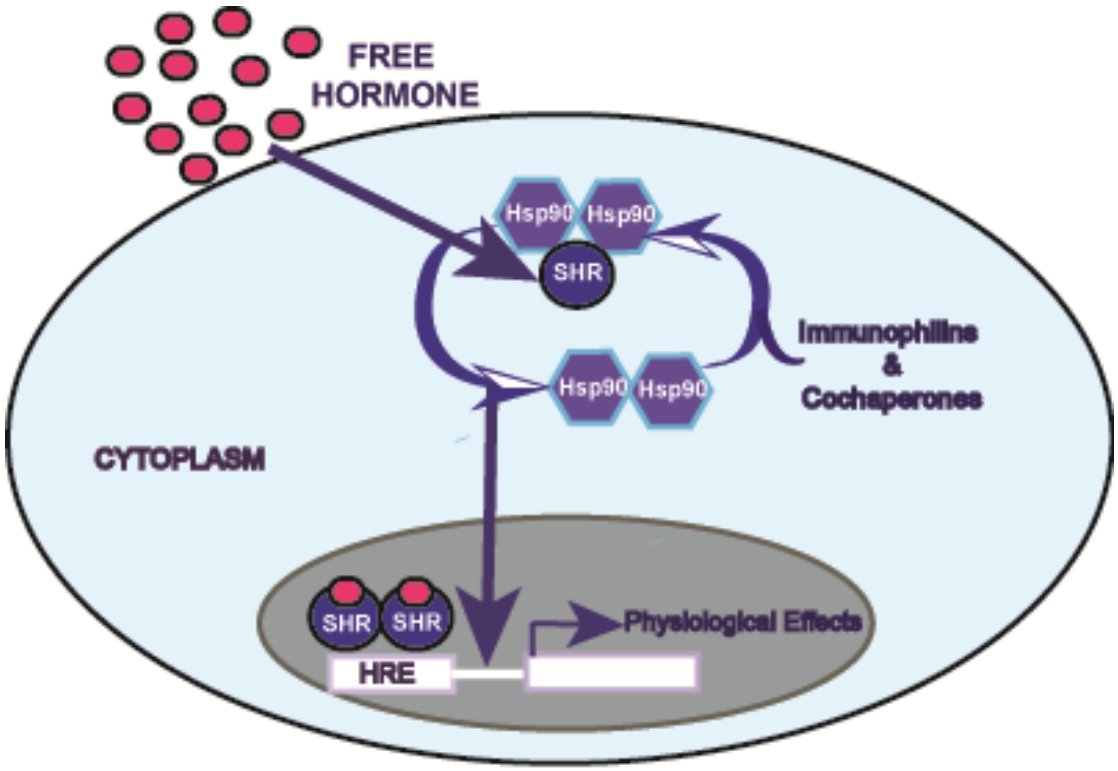



Figure 1.2: Steroid Hormone Receptor Signaling Pathway

Figure 1.2 is a depiction of the hormone-induced steroid hormone receptor signaling pathway. Hormone binds the mature steroid hormone receptor (SHR) complex, causing receptor dimerization and translocation to the nucleus, where the receptor binds response elements to activate transcription.

Transcription of isoform A can be initiated by the internal translation start site at the Met-188 residue. AR-B represents 91% of the AR species expressed in adult genital fibroblast tissue. AR-B contains a polyglutamine repeat tract that is absent in AR-A (15). AR-B exists in the cytoplasm as a receptor primarily to androgens and is also found in the nucleus where it serves as a transcription factor (2). In addition to isoforms A and B, castrate-resistant prostate cancer tissues display multiple AR splice variants and truncation mutants (16).

A proper investigation of putative interactions of the androgen receptor with other proteins requires an understanding of AR's structure. The androgen receptor has three main domains: the N-terminal domain, the DNA binding domain, and the ligand binding domain located at the C-terminus (2), as seen in **Figure 1.3**

The N-terminal domain (NTD) is the least conserved domain of the SHRs, with only 15% homology between the different receptors (2, 17). It contains the major activation site Activation Function 1 (AF-1). In the androgen receptor, the N-terminal domain contains a segment of polymorphic glycine and glutamate repeats. It also contains a FQNLF motif that allows the NTD to interact with the C-terminal domain (CTD), influencing trans-activation of the receptor (2). Additionally, AR contains the small ubiquitination like modifier (SUMO)ylation sites K386 and K520, and it is believed that SUMOylation of these sites causes transcriptional repression (18).

AR's DNA binding domain is a stretch of approximately 800 amino acids characterized by two zinc finger motifs. The DBD is highly conserved among the nuclear receptors. Therefore, the hormone response elements to which all nuclear receptors bind are also conserved (19, 20). AR binds DNA more tightly than any other nuclear receptor due to its higher affinity-dimerization (2). The first zinc finger of AR contacts the major groove of DNA, and this region


Figure 1.3: Domains of the Androgen Receptor

Major domains of the androgen receptor are highlighted. The regulatory regions and nuclear localization sequences are depicted below the representation of the corresponding AR domain.

of DNA is termed the proximal (P) box. The second zinc finger is responsible for ensuring that dimerization occurs stably in response to binding to DNA and that the dimer is in the proper orientation to contact the hormone response element (HRE); this region of the DNA is termed the distal (D) box (2, 5).

The hinge region is located between the DNA binding domain and the ligand binding domain and is important for cellular localization and degradation. This region contains the nuclear localization and export sequences. The androgen receptor associates with importin α for nuclear import (2). It appears that the immunophilin FKBP52 assists with nuclear import of the receptor in some cases (3, 21, 22). There is a potential proline, glutamic acid, serine, and threonine-rich (PEST) sequence in the hinge region of AR which may be involved in protein degradation pathways. Additionally, the hinge region undergoes post-translational modifications such as phosphorylation by Mitogen Activated Protein Kinase (MEK-K) kinases to assist in translocation and acetylation by p300/CBP-associated factor (pCAF), HIV-Tat Interacting Protein (Tip60), and CCAAT Binding Factor (CBF)/p300 (2). Acetylation of AR is associated with decreased activation of receptor, decreased cofactor recruitment to the promoter, increased recruitment of transcriptional co-repressors, and decreased nuclear AR localization (23).

The ligand binding domain of AR, as seen in **Figure 1.4,** is composed of eleven α-helices comprising an internal ligand binding cavity (20). The helices are named one through twelve (there is no second helix, however) to keep the nomenclature consistent between different receptors. The natural hormone ligands for the androgen receptor are DHT and testosterone (2). Ligand contacts 18 crucial residues of the ligand binding domain on helices 3, 5, and 11; this internal binding site is known as Binding Function 1 BF-1 [10, 22, 34-35]. Helix 12 is a gate on the ligand binding domain; it opens to permit ligand binding and closes once ligand has bound.

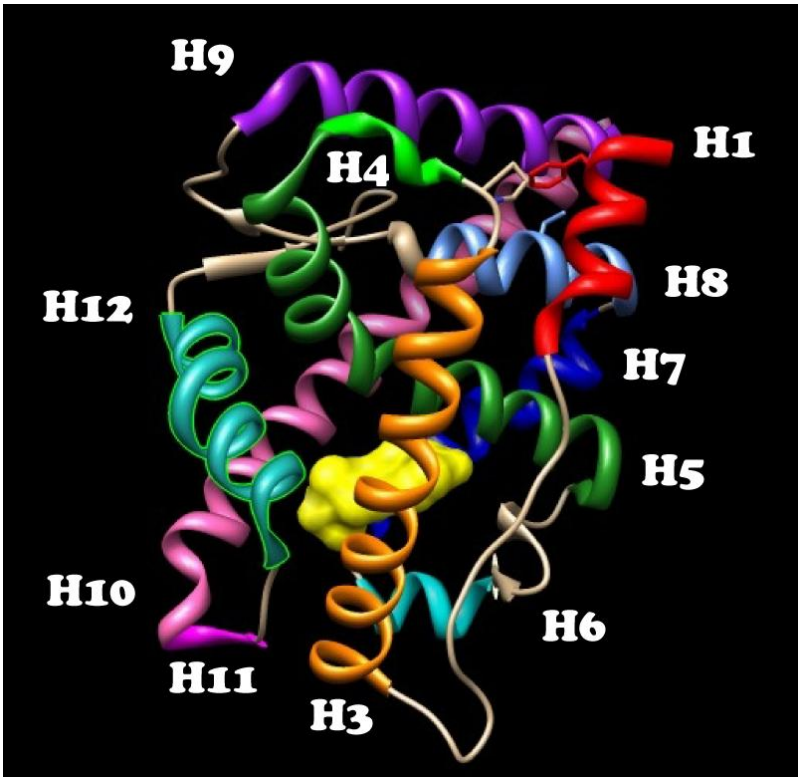



Figure 1.4: Ligand Binding Domain of the Androgen Receptor bound to R1881

Figure 1.4 is a graphic adapted from the Androgen Receptor Gene Mutations Database (http://androgendb.mcgill.ca/ARmodel.jpg), designed by Dr. JH Wu of the LDI Molecular Modeling Laboratory. The image was generated through UCSF Chimera Version 1.5 using PDB 1XQ3. Helices are labeled 1 through 12 and appear in different colors. The bound synthetic agonist R1881 (ball and stick) appears in yellow. The LBD is oriented to the BF3 surface. The key residues of the putative FKBP52 binding surface F673(red), P723 (tan), and C806 (cornflower blue) project from the BF3 surface as seen in the figure.

Once ligand binds, a hydrophobic region known as Activation Function 2 (AF-2) surfaces on the ligand binding domain, allowing receptor dimerization (24) and recruitment of coactivator binding via LXXLL binding motifs (2, 25, 26). Furthermore, the FXXLF motifs of alpha helices in nuclear receptor (NR)-boxes of coregulators such as the steroid receptor coactivator 2 (SRC2) and AR Associated Protein 70 (ARA70) also facilitate binding to this surface (27, 28).

Further study of the role of the receptor, its coregulators, and modifications to the androgen receptor at the post-transcriptional and translational levels are all topics for study in endocrine receptor research.

### 1.3 HSP90

Hsp90 is a chaperone with more than 200 client proteins, including steroid hormone receptors, stress response proteins, and cellular cascade members. Hsp90 complexes with Hsp70 and co-chaperone proteins to form the dynamic Hsp90 chaperone machine. The function of Hsp90 as a chaperone can be described in three parts: assisting in the folding of proteins, resisting aggregation, and participating in the association and assembly of multimeric proteins such as steroid hormone receptor complexes. Hsp90 functions to maintain cellular homeostasis, to drive heat shock response via activation of Heat Shock Transcription Factor 1 (HSF1) activity, and even to regulate transcriptional events (29).

A monomer of Hsp90 consists of three domains. The N-terminal domain contains an ATP binding site, which is critical for chaperone function. The middle domain contacts client proteins and also is responsible for ATPase activity. The C-terminal domain contains the motif by which the chaperone protein dimerizes. The current model for Hsp90 chaperoning suggests that Hsp90 begins in an open state in which the N-terminus does not have a nucleotide present, and when ATP binds each of the N termini, it favors a shift of conformation, promoting an

interaction of the N termini together. Meanwhile, co-chaperones HOP or cell division cycle 37 homologue (CDC37) can bind and temporarily delay the N terminal dimerization. These proteins deliver client proteins such as steroid hormone receptors and protein kinases, respectively. As Hsp90 dimerizes at the N-termini, it adopts a closed and contorted orientation. Activator of Hsp90 ATPase1 (AHA1) binding promotes a favorable conformation of the chaperone dimer for ATP hydrolysis. The p23 co-chaperone stabilizes this closed conformation of the dimer. ATP is then hydrolyzed by the middle domain, and the ADP dissociates, triggering the opening of the Hsp90 dimer for the binding of the next client protein (29). **Figure 1.5** gives a graphical depiction of the Hsp90 chaperoning cycle.

Hsp90 regulation can occur naturally via interactions with co-chaperones and post-translational modifications. The co-chaperones of Hsp90 include but are not limited to HOP, p23, Small Glutamine Rich Tetratricopeptide Repeat Protein 1 (SGT1), FKBP51, FKBP52, CDC37, AHA1, Serine/Threonine Protein Phosphatase 5 (PP5), CHIP, Tetratricopeptide Repeat-containing Protein associated with Hsp90 (TAH1) and protein interacting with Hsp90 (PIH1). The effects of co-chaperone binding to Hsp90 are diverse, from delivering client proteins, assisting in chaperoning activity via modulation of ATPase state, increasing functionality of client proteins, decreasing client protein functionality, and promoting client protein degradation. The dynamic interaction of Hsp90 with the net sum of its clients and co-chaperone proteins determines the regulatory outcome of the interaction with client protein (29).

Post-translational modifications of Hsp90 include phosphorylation, acetylation, and S-nitrosylation. Phosphorylation of Hsp90 is associated with Vascular Endothelial Growth Factor 2 (VEGF2)-induced angiogenesis, apoptosome formation, and kinase chaperoning.

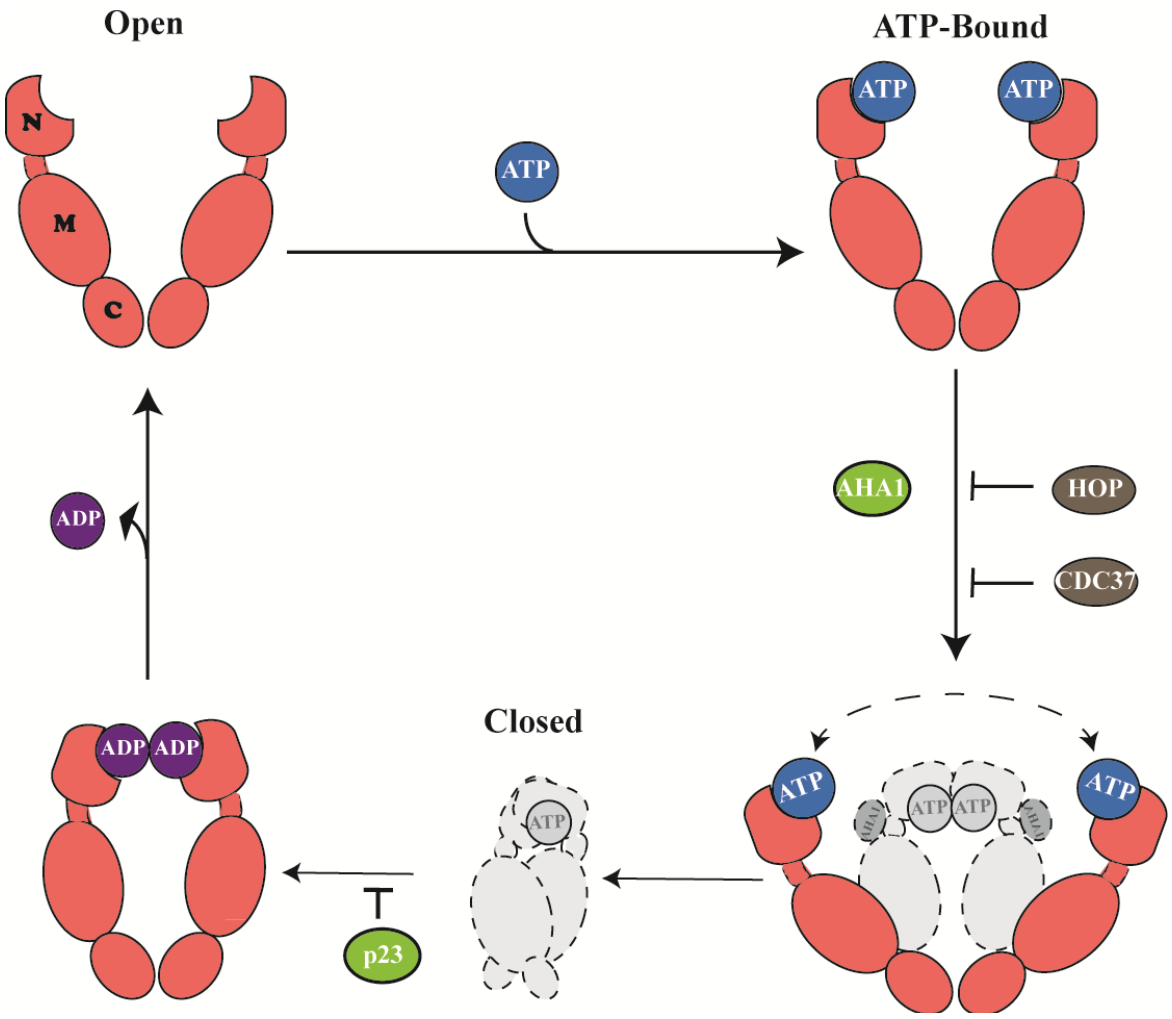



Figure 1.5: Hsp90 Binding Cycle

Figure 1.5 is a graphic modified from Figure 1, *Nature Reviews Cancer* **10**, 537-549 (August 2010) | doi:10.1038/nrc2887. The Hsp90 dimer's open conformation allows ATP to bind to the N terminals of the protein, inducing association of HOP or CDC37 to deliver client proteins and temporarily delay the clamping of the dimer's N termini. AHA1 binding favors movement towards ATP hydrolysis. P23 stabilizes a closed dimer conformation so that client protein is maintained in association with Hsp90. ATP is hydrolyzed to ADP by the middle domain, and ADP dissociates, allowing Hsp90 to adopt the open conformation once again.

It appears that acetylation is linked to effective binding of Hsp90 to p23, ATP, and client proteins. S-nitrosylation can inhibit ATPase activity. (29).

Hsp90 has a well-established role in controlling cellular distribution of steroid hormone receptors, in promoting ligand binding, and in helping to stabilize the complex (29). Some argue that Hsp90 may also play a role in the nucleus. Indeed, three percent of all Hsp90 exists in the nucleus (30). Hsp90 is known to interact at the nuclear level with B Cell Lymphoma 6 Protein (BCL-6) and the proteins SET and MYND domain containing 2 and 3 (SMYD-2 and 3). Hsp90 binds BCL-6, forming a transcriptional repressor complex in the nucleus (31). Activity of the histone methyltransferases SMYD-2 and SMYD 3 increases in the presence of their binding partner Hsp90 in vitro (32). Both SMYD-2 and SMYD-3 expression is increased in several cancers (32, 33).

### 1.4 FKBP51 AND FKBP52

The immunophilins are a class of proteins, which are distinguished by their ability to bind immusuppressive drugs through PPIase domains. Immunophilins can be further separated into two categories according to immunosuppressive drug binding. Cyclophilins (CyP) bind the drug cyclosporin-A, and FK506 binding proteins (FKBPs) bind rapamycin and FK506. The FKBPs contain varying numbers of FK domains, and a subset binds Hsp90 (10).

Two members of the FKBP family which possess Hsp90 binding abilities are FKBP51 and FKBP52. FKBP51 and FKBP52 are 60% identical and approximately 70% similar in amino acid conservation (34). **Figure 1.6** shows a structural comparison of FKBP51 and FKBP52. The proteins contain the same structural domains but differ visibly in the orientation of the tetratricopeptide repeat (TPR) domains as seen through the crystal structure. The FK domains are known as FKBP12-like domains.

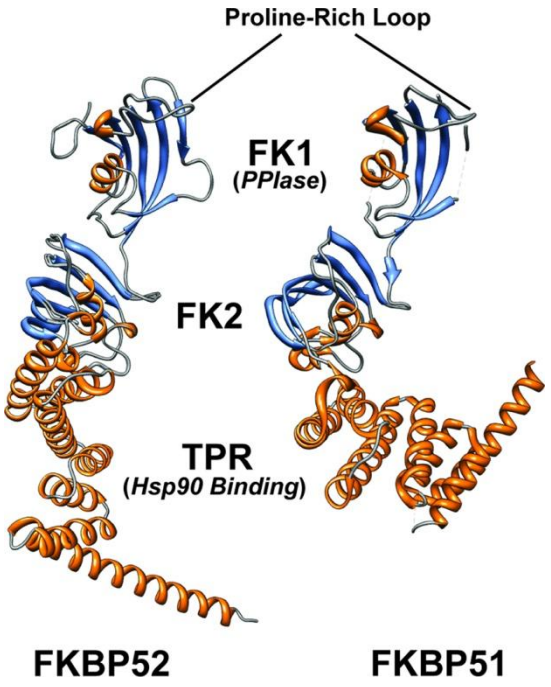



Figure 1.6: Structural Comparison of FKBP51 and FKBP52

X-ray crystallographic structures of FKBP51 and FKBP52. The three-dimensional structure of the composite of two partial structures for human FKBP52 (protein databank numbers 1Q1C and 1P5Q) and the structure of human FKBP51 (protein databank number 1KT0) are shown in ribbon format. Beta pleated sheets are in blue and alpha helices are in orange. The important functional regions FK1, FK2, and TPR are illustrated. The figure was created, including overlaying the two partial FKBP52 structures, using UCSF Chimera version 1.5. It appears as Figure 1 in *Trends in Endocrinology Metabolism,* 2011 December; 22(12): 481–490.

FK506 is the immunosuppressive drug which can bind any FKBP12-like domain but only initiates immunosuppressive activity due to calcineurin inhibition through FKBP12 (35).

In both FKBP51 and FKBP52, the FK1 domain has functional peptidyl-prolyl cis-trans isomerase (PPIase) activity and can bind the immunosuppressant FK506, whereas the FK2 domain is a PPIase-like domain with unknown function that lacks PPIase activity. FK1 regions are conserved between the proteins, with the exception of a few amino acids at the proline-rich loop of the PPIase domain. The hinge region connects FK1 to FK2 and is thus called the FK loop. In FKBP52, the hinge region possesses a TEED phosphorylation site which is absent in FKBP51 (10). Casein kinase II modifies this site at T143, which appears to be a regulation point of FKBP52-receptor potentiation. It appears that when the phosphorylation-resistant mutant T143E is constructed in FKBP52, the ability of FKBP52 to potentiate the action of receptors is decreased but not completely abolished. It is believed that this mutation impacts the ability of FKBP52's linker region to form Hydrogen bonds properly (36). Lastly, the TPR domain, characterized by helix-loop-helix motifs in triplicate, is responsible for Hsp90 binding. 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. FKBP51 exhibits ionic bonding between the FK2 and TPR domains, whereas FKBP52 lacks these bonds and is therefore more flexible in nature (10).

At the animal level, much of our knowledge of FKBP51 and FKBP52 was gained through studies of knockout mice. Male FKBP52 knockout mice (52KO) show varying degrees of hypospadias, irregularities of genitalia, nipple enlargement, decreased size and abnormal appearance of seminal vesicles and of the anterior prostate gland, and problems with testes descent. Also, 52KO mice exhibit increased levels of corticosterone, although they circulate

normal levels of androgens. The physiological abnormalities of the mice parallel those of human pathologies in Androgen Insensitivity Syndrome. Male 52 KO mice are infertile, primarily due to compromised AR signaling. Dey and colleagues noted decreased spermatozoa motility, decreased spermatozoa count, and decreased fertilization efficiency in male mice (37). 52 KO female mice are infertile, although they lack external abnormalities and exhibit normal oogenesis (11). Female infertility appears to be due to compromised PR signaling, thereby inhibiting embryonic implantation in the uterus. 52 KO mice show only half the binding affinity of PR for progesterone as wild-type mice (13). Interestingly, 52 KO mice exhibit 50% embryonic lethality (11). On the other hand, FKBP51 KO mice do not exhibit physiological phenotypes from gene deletion. However, when FKBP51 (*fkbp5)* and FKBP52 (*fkbp4)* are both knocked out, it results in 100% embryonic lethality (38). This can be attributed to some redundancy of function of FKBP51 and FKBP52.

The tissues of the 52KO mice were also sectioned and examined to further characterize pathology and were compared to normal mice. FKBP52 was expressed in several wild type mouse tissues but not in 52 KO mouse tissues. Histological sections revealed that AR was still present in the nuclei of Leydig, peritubular myoid cells, and Sertoli cells of the testes, but AR expression was decreased significantly in the sections. Western blots revealed that the expression profiles of other immunophilins (FKBP51, PP5, and Cyp40) were not significantly changed due to the absence of FKBP52 in 52 KO mouse tissues, although AR expression decreased slightly (11). In another study by the Dey group, 52KO female mice exhibited progesterone resistance in the uterus. When endometriotic uterine tissues were transplanted in both the wild type and knock down mice, lesion growth increased, inflammation augmented, and

more angiogenesis was seen in 52 KO mice over wild-type mice; these are characteristic of defects in decreased PR signaling (39).

Additional studies in human tissues correlate findings from mouse models. Studies indicate that FKBP52 and AR colocalize in the epithelium of luminal prostate tissue, but FKBP52 appears absent in stromal cells (11). FKBP52 expression is decreased in human endometrial tissues of endometriosis patients (39). Its over-expression in human prostate needle biopsies of prostate cancer patients has named it a potential biomarker of prostate cancer (40).

Studies at the molecular level also suggest that the immunophilins (particularly FKBP51 and FKBP52) regulate receptors by different mechanisms. They bind Hsp90 by different means; FKBP52 requires solely the TPR domain for binding, yet FKBP51 requires additional sequences up and downstream of the TPR domain. Furthermore, FKBP52 binds FK506 without detaching from a GR complex, yet FKBP51 detaches from the complex when it binds FK506 (41). Additionally, the Hsp90 client protein (receptor) seems to influence the recruitment of some immunophilins over others. While FKBP51 and FKBP52 compete with equal preference for binding to Hsp90 in a purified system with the client PR, they do not appear to have equal competition *in vivo*. In a cell-free PR system using reticulocyte lysate (RL), a cell-free extract containing all the cochaperones and chaperones necessary for receptor assembly, the ratio (in moles) of Cyp 40: FKBP52: FKBP51 was 10:5:1, yet FKBP51 appeared to associate with PR complexes up to four times as much as compared to that of FKBP52 and Cyp40 (42). Furthermore, GR recruits FKBP51 or Cyp40, and ER recruits Cyp40 preferentially (38). This suggests the possibility that either these immunophilins not only bind Hsp90 but also bind the receptors directly, or that there are additional, uncharacterized mediators of the individual receptors, which enforce the preferential recruitment of immunophilins.

Although FKBP51 and FKBP52 have only a few structural differences, their divergent functions are still being characterized at the cellular level. FKBP52 can potentiate the action of GR (12), PR (13), and AR (11) but not ER or MR (12) as transcription factors. FKBP51, on the other hand, actually causes a decrease in GR binding to glucocorticoids in squirrel monkeys (43). Riggs et al. showed that FKBP51's apparent inhibition of GR signaling was actually an inhibition of FKBP52 signaling through the use of a yeast-based reporter assay. By substituting FKBP52 for FKBP51 in a transfection involving GR and reporters, a three-fold leftward shift in potency of a dose response curve for GR was seen. They examined hormone binding affinities of GR in the presence of FKBP51 or FKBP52 and the rest of the Hsp90 steroid hormone receptor complex. GR's hormone binding affinity for deoxycorticosterone (DOC) was up to five times greater with FKBP52 than with FKBP51. Additionally, in an *in vitro* study, it appeared that FKBP51 decreased PR's response to hormone (12). An increase in progesterone levels seems to enhance FKBP51 expression; feasibly FKBP51 could serve as a negative regulator of PR-related pathways (44). FKBP51 is regarded as a repressor of hormone receptor signaling (10). In fact, FKBP51 often was used as the negative control for AR transcriptional studies with FKBP52 (45).

On the other hand, in the human prostate cancer cell line PC-3, FKBP51 exhibits positive regulation of AR by increasing its binding affinity for androgen and enhancing the amount of p23 recruitment to the AR complex. However, for the purposes of this recent study, the synthetic hormone concentration reached potentially non-physiological levels of 1nM R1881, a mutant receptor was studied, and multiple prostate cancer pathways could have been activated (46). More research must be conducted to draw any definitive conclusions regarding the

mechanisms and roles of both FKBP51 and FKBP52 in regulating steroid hormone receptors, both in pathological and healthy tissues.

One theory regarding immunophilin regulation of receptors was that they could interact with receptors through a mechanism involving the receptors' DBDs. Through domain swapping experiments of ER (which lacks FKBP52 potentiation abilities) and GR, they found that the LBD of the receptor was responsible for regulation by FKBP52, not the DBD (12).

Other studies focused on the immunophilins themselves to characterize their interactions and functional alterations in conjunction with steroid hormone receptor complexes. The Smith lab found that an FKBP52-K354A mutation (a TPR mutation which had previously been found to disrupt Hsp90 binding) prevents full FKBP52-induced potentiation of AR in a yeast reporter system, verifying that Hsp90 binding is important for potentiation of the receptor (11). When the FK1 domains of FKBP51 and FKBP52 were switched, they found that the chimera with an FKBP52 backbone completely lacked potentiation abilities with GR. However, the chimera with the FKBP51 backbone gained up to 50% of the potentiation effects of wild-type FKBP52, suggesting that although the FK1 domain is a crucial component of potentiation, it is not the sole determinant of FKBP52's abilities to augment GR signaling (12).

PPIase activity is associated with the FK1 domain, and very early studies of FKBP52 showed that FK506 (which binds FK1) blocked FKBP52's PPIase activity (47). The Smith group determined that administration of FK506 prior to addition of hormone effectively prevented FKBP52's potentiation of the receptor. However, FK506 is a large drug, so it was important to verify that steric hindrance was not the reason for potentiation changes. The highly conserved residues F67 and D68 of the PPIase domain were mutated to form the FD67DV mutant (12). Previously this mutant was constructed in FKBP51, and it was associated with a

90% decrease in PPIase activity without affecting association of FKBP51 in PR complexes (41). When the FKBP52-FD67DV mutation was constructed, potentiation of GR was prevented in a yeast model (12). However, it only partially decreased AR signaling in a yeast model. Still, there was still a chance that even this mutation compromised the conformation of FKBP52, so the mutations F67Y, W90L, and F130Y were constructed, which abrogated PPIase activity without severely altering the conformation of the PPIase domain itself. These mutations showed no significant change in wild-type FKBP52-enhanced GR and AR activity in yeast reporter assays, and the potentiation of AR by FKBP52 was also seen in the 52KO MEF model (45).

Initially, when PPIase activity was deemed critical for receptor potentiation by FKBP52, researchers searched for prolines in AR, which could serve as potential substrates for PPIase activity. The AR mutant P723S, found in androgen sensitivity syndrome patients, was one such mutant of interest. Surprisingly, it actually did not appear to be a substrate for the PPIase domain but was interesting for another reason: its dependence on FKBP52 for functionality. Receptor signaling is significantly decreased with this mutant, but signaling is restored in the presence of FKBP52 (11).

The significance of the PPIase domain was finally determined through random mutagenesis of FKBP51. The AR mutant P723S could be exploited for use in a genetic approach to characterize FKBP52's functional determinants of potentiation. A library of FKBP51 mutants, which had gained FKBP52-like potentiation abilities towards the FKBP52-dependent AR P723S, was created. Random mutagenesis of FKBP51 revealed three consistent mutations, which were associated with FKBP52-like potentiation of AR. Site-directed mutagenesis was performed to generate these mutants, and the mutants were examined individually with wild-type GR and AR in receptor-mediated yeast reporter assays and then with

52 KO MEF luciferase assays. When the FKBP51-L119P mutant was assayed in 52 KO MEFs, it increased receptor potentiation up to five times that of wild type FKBP51. When the converse mutation was made in FKBP52, the P119L mutant only possessed 50% of the potentiation ability of wild type FKPB52. The double mutant FKBP51-A116V/L119P exhibited increased AR signaling up to twenty times higher than wild-type FKBP51, thereby rivaling FKBP52's potentiation abilities in both yeast and 52 KO MEFs. These mutations alter the conformation of a loop, which is suspended above the PPIase pocket in the FK1 domain (45).

Position 119 within the proline-rich loop directly above the PPIase pocket of the FKBPs differs between FKBP51 and FKBP52. In FKBP52, the amino acid is a proline, and in FKBP51, it is a leucine. Due to the characteristics of these amino acids, in FKBP52, the loop projects outward and is more accessible. However, the leucine residue causes FKBP51 to adopt a more closed conformation of the loop. Nonetheless, the presence of a L119P mutation alone does not confer full receptor potentiation ability to FKBP51. This suggests that other residues could be involved in generating FKBP52's potentiation of receptor. FKBP51/FKBP52 chimera studies in yeast and 52 KO MEFs revealed that while the FK1 domain seems responsible for the majority of FKBP52's potentiation, FK2 and TPR domain interactions may also play a role in this characteristic potentiation of the AR (45).

Since there have been reports of interactions of FKBP52 with dynein (48), the Smith group examined GR potentiation by FKBP52 in a dynein-deficient yeast strain, and they found that FKBP52 exerted similar potentiation of GR signaling independently of a dynein interaction (12). Originally, it was proposed that the immunophilins PP5, CyP40, and FKBP52 tethered the receptor to dynein to facilitate retrograde transport of the complex (22), and later it was suggested that FKBP51, which does not appear to bind dynein, suspends the receptor complex in

the cytoplasm until it is an appropriate time for translocation (49). Additionally, FKBP52 has been implicated in further regulation of receptor compartmentalization by binding tubulin to prevent its polymerization into microtubules (50). An argument can be made that all the studies conducted which have demonstrated these interactions have involved purified protein fragments rather than full length proteins, and none of those studies confirm direct, correlative roles for FKBP52 in cellular localization *in vivo* (22, 49-52).

### 1.5 B-CATENIN

β-catenin, a 92 kDa protein, is involved in many processes, from signal transduction to cellular adhesion (53, 54). It is a well-characterized member of various pathways such as the phosphatidyl inositol-3'-kinase (PI3K)/Akt pathway, insulin growth factor 1 (IGF-1) pathway, and Wingless/Int (Wnt) pathways, ultimately leading to transcription of targets such as the protooncogene c-myc, cyclin D, matrix metalloprotease 7 (MMP-7), endothelin 1, cyclooxygenase 2 (COX-2), survivin, and peroxisome proliferator activated receptor γ (PPARγ). In many instances, β-catenin acts as a coactivator of transcription. When stimulated by the secretory glycoprotein Wnt, it interacts with Ternary Complex 4 (TCF4) and Lymphocyte Enhancement Factor 1 (LEF-1) transcription factors to regulate growth and development genes. Alternatively, it can associate with AR in response to Wnt stimulation (55-57). β-catenin plays roles in cell proliferation, apoptosis resistance, cellular migration, carcinogenesis, and differentiation (58-60). Due to its pervasive nature, this protein has widespread implications for various types of cancers affecting the ovaries (61), bladder (62), colon (63), breast (64), and prostate (65).

The Wnt signaling pathway poses several regulatory mechanisms which malfunction in prostate cancer development. In non-cancerous tissues, β-catenin generally associates with the

cytoplasmic membrane. Sequestered by the transmembrane epithelial cell protein E-cadherin, β-catenin is unavailable for nuclear translocation and transactivation of transcription factors such as LEF-1 (66). Instead, it participates in calcium-dependent cellular adhesion mechanisms via adherens junctions. When β-catenin associates with E-cadherin, the complex actively organizes and tethers microfilaments in the cell. Meanwhile, E-cadherin's extracellular portion engages cadherins of adjacent cells to maintain cellular adhesion (54, 67). β-catenin associates with E-cadherin to modulate intracellular and intercellular signaling events and to regulate transcriptional events (67, 68). Any unbound β-catenin is generally tagged for destruction by the degradation complex, which consists of Glycogen Synthase Kinase 3 β (GSK3β), the tumor suppressor Adenomatous polyposis coli (APC), the scaffolding protein axin, and casein kinase 1 (69).

The classic Wnt pathway states that Wnt activation of the Frizzled receptor deactivates the degradation complex member GSK3β, thereby enabling cytoplasmic β-catenin to evade ubiquitination due to its de-phosphorylated status. Wnt induction of the receptor allows translocation of free β-catenin to the nucleus for regulation of gene expression (70). **Figure 1.7** shows a summary of the Wnt pathway.

In prostate cancer, β-catenin participates in cytoplasmic and nuclear events due to Wnt induction. Wnt inhibition is decreased and Wnt expression increases in prostate cancer. Expression of the natural Wnt inhibitor, Wnt inhibitory factor 1 (WIF1), is often decreased in prostate cancer cells (71), and in particular, expression of the β-catenin stabilizing factors Wnt 1, 2, and 5a are up-regulated in these cells (72).

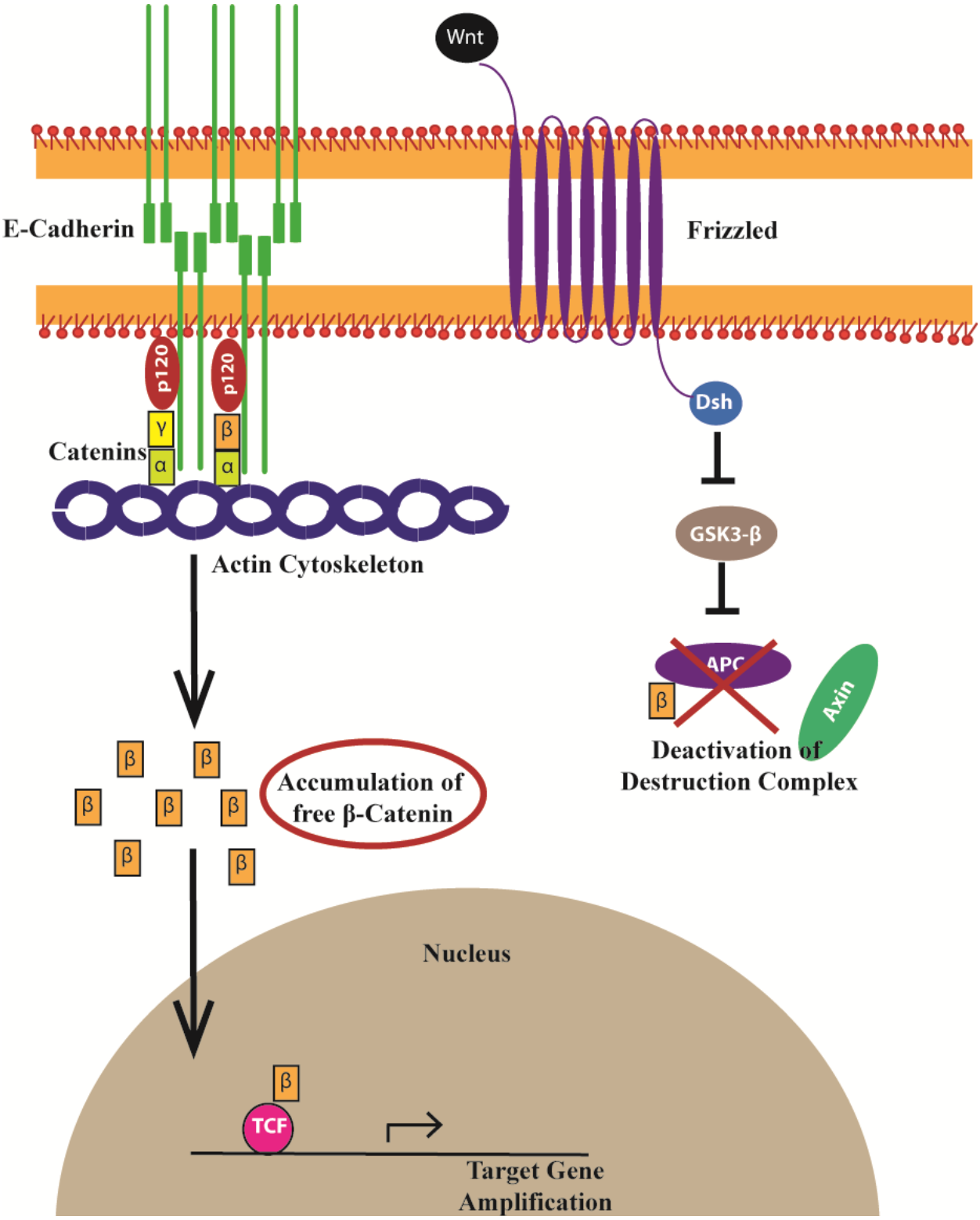



Figure 1.7: Wnt Pathway Signaling

Figure 1.7: Wnt Pathway Signaling

In the absence or presence of Wnt protein, β-catenin associates with E-cadherin near the cytoplasmic membrane and helps to tether E-cadherin to the actin cytoskeleton. Wnt signaling is initiated when Wnt binds the Frizzled receptor, leading to the recruitment of Disheveled protein to the membrane. Therefore, GSK3β does not activate the destruction complex, and cytoplasmic β-catenin accumulates and is available for nuclear roles as well. In the absence of Wnt, any free cytoplasmic β-catenin is tagged for destruction by the GSK3β/APC/axin complex. This is an adaptation of Figure 1 of *Journal of Biomedicine and Biotechnoogy*. 2011; 2011: 567305.

When LNCaP prostate cancer cells are treated with conditioned media with Wnt 3a, the result is cell growth and colony formation, which is associated with increased prostate specific antigen (PSA) transcription regardless of AR ligand presence or absence (73, 74). Androgen-independent, invasive PC-3 and DU145 prostate cancer cell lines exhibit higher amounts of Wnt/β-catenin signaling than androgen-dependent LNCaP cells or normal prostate cells (75).

In prostate cancer, β-catenin dissociates from E-cadherin due to debilitation of members of the destruction complex. Phosphorylation-defective β-catenin mutants are found in a small number of prostate cancer patient tissues (76), but they are not found in the major prostate cancer cell lines (77). APC, another member of the degradation complex for β Catenin, can be deactivated by post-translational modifications. By deactivating β-catenin's nuclear antagonist, β-catenin is thereby available and capable of regulating transcription (78). β-catenin expression is up-regulated in prostate cancer cells, and a greater percentage of β-catenin is found in the nucleus in cancerous cells as compared to normal cells (72). In metastatic prostate cancers, E-cadherin expression is decreased, resulting in a greater proportion of cytoplasmic and nuclear β-catenins (79). Thus, the destruction of E-cadherin promotes β-catenin signaling via the Wnt pathway.

Increased transcription of AR-target genes is a hallmark of prostate cancer (80). One study suggests that β-catenin modulates AR's requirement for ligand binding, thereby inducing AR hypersensitivity to circulating androgens (81). Thus, β-catenin could play a role in transitioning prostate tissues from a hormone-dependent to a hormone refractory stage when AR is constitutively activated, regardless of concentration of circulating hormone. Wnt signaling involving β-catenin is implicated in two main areas of androgen receptor (AR) regulation. In the direct pathway, β-catenin is a co-activator of AR-mediated transcription (82). In the indirect

pathways, β-catenin participates in the regulation of AR signaling via its interaction with other pathways. For example, Wnt stimulation deactivates GSK-3β, resulting in a wide range of effects on AR in its hyperphosphorylated state. However, in these pathways β-catenin itself never contacts the receptor directly to change its phosphorylation status (83, 84). Studies of AR(+) LNCaP and AR(-) PC3 cells using time-course fractionation and localization studies suggest that AR participates in transporting β-catenin to the nucleus in response to the hormone agonist R1881, and that β-catenin is present in the complex even at the point of AR binding to hormone response elements (85).

In 2003, the Gelmann lab characterized some of the critical residues of AR, which participate in binding to β-catenin. In these studies, β-catenin bound the receptor near the AF-2 domain of the LBD in experiments employing a mammalian two-hybrid binding model. While β-catenin was able to bind the receptor in the absence of ligand, binding increased tremendously in the presence of the hormone agonist R1881. When a K720A mutation was made in helix 3 of AR, the binding of β-catenin was disrupted. However, the mutation simultaneously decreased the binding affinity of the NTD:LBD interaction of the receptor and additionally decreased the incidence of the P160 coactivator transcriptional mediators/intermediary factor 2 (TIF-2)'s binding to the receptor. This suggests that, rather than being the main determinant of β-catenin binding, this mutation may merely compromise the conformation of AR. Additionally, several amino acids at positions 891-902 on helix 12 of AR were mutated, resulting in a wide range of effects on hormone binding as well as alterations in binding to β-catenin, TIF-2, and transactivation of the receptor. Particularly, they found that a mutation of AR at residue E893A of helix 12 caused a huge decrease in binding to β-catenin, a smaller decrease in binding to the NTD of the receptor, and only a minor decrease in binding to TIF-2 (81).

Labs have also attempted to determine critical determinants of β-catenin binding to AR, but there was less success at pinpointing exact residues. The armadillo repeats of β-catenin appear to be crucial for its interactions with AR, as found through yeast two-hybrid studies (82) as well as glutathione-S-transferase (GST) pull-down assays (85). GST-pull-down assays by the Gelmann lab showed that androgen receptor LBD pull-down utilizing full-length β-catenin protein as bait was strengthened with R1881 addition. When a fragment of β-catenin's C-terminal domain was used containing armadillo repeats 8-12, β-catenin bound AR LBD regardless of hormone concentration. When a N-terminal β-catenin fragment comprised of repeats 1-3 was used, very little AR LBD pulled down, but R1881 addition increased the interactions. Because β-catenin has five NR boxes, they theorized that maybe the interaction motifs in helices 1, 3, 7, 10, and 12 were responsible for the β-catenin/AR interaction, but individual mutations to these motifs did not affect AR binding. To see if β-catenin's interaction with AR could be prevented, the protein inhibitor to β-catenin and TCF-4 (ICAT), which competitively binds β-catenin at the armadillo repeats used to bind TCF-4, was employed. It successfully disrupted β-catenin/AR interactions as well (81). This could suggest similarities of binding sites between AR and TCF-4. More studies are needed to characterize β-catenin/AR interactions.

## 1.6 PROSTATE CANCER

Cancer is undeniably one of the most challenging universal health problems. One in two men and one in three women will face cancer in their lives. The number of cancer survivors increases steadily due to better medical care as well as increased life expectancy. In a survey on January 1, 2012, 13.7 million Americans reported a history of cancer, and the three most

prevalent cancers among the male population were melanoma (7%), colorectal (9%), and prostate (43%) (86).

Prostate cancer is an undeniable concern of many men in the US. According to the National Cancer Institute (NCI), one out of every six men in the US will develop prostate cancer (87). Prostate cancer is the most commonly diagnosed tumor and the second leading cause of cancer-related deaths in the US (88). It is the second most common cancer in men, second to skin cancer. The American Cancer Society (ACS) stated that in 2012, it was predicted that 241,740 people would be diagnosed with prostate cancer, and approximately 28,170 people will die due to the disease (89). In the 2009 Surveillance Epidemiology and End Results study sponsored by the NCI, it was determined that the median age of prostate cancer patients is 67, and the median age of death is 80 (87).

There are virtually no symptoms in early stage prostate cancer, but some patients develop pre-cancerous conditions such as benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN). In BPH, a natural consequence of age, the prostate tissue near the urethra continues to grow, restricting the flow of urine and semen. In PIN, prostate cells begin to appear abnormal as sampled but do not express biomarkers of cancer or tumor formation (89).

Prostate cancer is commonly diagnosed through the biopsy Gleason score, the extent of the primary tumor via the digital rectal exam, and serum PSA levels. Risk of reoccurrence is calculated based on the overall results of these three tests (88). Although PSA screening has been a standard recommendation for older men and indeed resulted in more frequent diagnoses of prostate cancer since its introduction in the 1990s (90), it has been questioned as a valid measurement of cancer burden when used alone. According to the ACS, "At this time, there are insufficient data to recommend for or against routine testing for early prostate cancer detection

with the PSA test." The risk factors in prostate cancer are age, race, and family history. It is possible to have prostate cancer and have no symptoms, but when a patient presents at the advanced stages of disease, the chief complaints are painful or discontinuous urination and the presence of blood in urine (91). Additionally, 68% of advanced prostate cancer patients will experience bone metastasis (86).

Castration-resistant prostate cancer (CRPC), also referred to as androgen-independent prostate cancer, is cancer that continues to develop despite low levels of serum testosterone as seen in castration. Recently, scientists have determined that the term androgen-independent is a misnomer; cancer patients who have CRPC are actually hypersensitive to trace levels of hormone (92). Generally, CRPC emerges in 10-20% of prostate cancer patients approximately five years after beginning prostate cancer treatment (93). When treatment involves androgen deprivation therapy, CRPC progression occurs between 1 to 2 years later, with a median survival rate of 1-2 years following onset of CRPC. CRPC can progress in spite of castration or hormone therapy into a stage termed hormone refractory prostate cancer (HRPC) in which it is no longer responsive to any types of hormone therapy (89). This state is possibly due to AR amplification, AR point mutations, AR coactivator overexpression, AR repressor underexpression, hormone independent splice variants, epigenetic alterations, and increased adrenal or intratumoral androgen production (94, 95). In CRPC and HRPC, PSA levels typically increase, indicating that AR signaling pathways are activated (94), but in some cases, the prostate cancer progresses even in the absence of increased serum PSA (96).

### 1.7 PROSTATE CANCER TREATMENTS

The manner by which prostate cancer is treated depends on the age of the patient, the stage of the cancer, and the desires of the informed patient. In less advanced cases of prostate

cancer, 57% of patients 65 and younger choose radical prostatectomy. In patients 65 to 74, 33% are treated in this manner, and 42% are treated by radiation. Advanced stage and metastatic patients are treated by androgen deprivation therapy (ADT), radiation therapy, bone-metastasis therapy, or a combination of treatments. The most common side effect of ADT treatment is sexual difficulty, present in 80% of patients (86).

### 1.7.1 Conventional Prostate Cancer Treatments

Early stage prostate cancer patients typically are treated through radiation or surgery. In radiation treatments, either external beam radiation exposure is used or radioactive seeds are implanted into the prostate. Alternatively, the prostate can be removed (91). Both treatments appear to have similar efficacy. Side effects of surgery are incontinence, lymphedema, impotence, and decreased fertility. Side effects of radiation treatment are bowel problems, incontinence, and fatigue (89).

Late stage, metastatic prostate cancer patients are treated through androgen deprivation therapy. Hormone therapy has been used to treat these patients for greater than 70 years, but it is still a controversial therapy (94). ADT's goal is to reduce the circulating DHT concentration to 20 ng/dl or less, which is about 80% of many of the CRPC patients' circulating hormone concentration (97). ADT can entail castration (orchiectomy) and/or the use of therapeutics such as CYP17 inhibitors, luteinizing hormone releasing hormone (LHRH) agonists or antagonists, or anti-androgens (89, 97). The CYP17 inhibitors such as ketoconozole and abiraterone prevent the conversion of C21 steroids into C19 adrenal androgens. LHRH agonists and antagonists interfere with the production of DHT at a more preliminary level of the pituitary-testicular axis (97). The problem with LHRH agonists is that initially, they cause a surge of testosterone as they stimulate leutenizing hormone (LH) and follicle stimulating hormone (FSH), but within 4-6

weeks, the LHRH receptors are down-regulated in the presence of excess hormone, and then testosterone will actually decrease to castration levels (91). LHRH antagonists prevent the downstream production of testosterone by blocking the production of LH and FSH (89). LHRH agonists and antagonists do not prevent production of DHT through the adrenal gland, which is responsible for 5-10% of circulating androgen.

Non-steroidal anti-androgens were created to address this problem (94). Non-steroidal anti androgens such as Bicalutamide (Casodex; Astra Zeneca, London, U.K.) and Flutamide (Eulexin; Schering, Kennelworth, N.J.) compete with hormone in binding the AR LBD selectively but do not induce wild type (wt) AR-mediated transcription (98). However, when AR is over-expressed or a mutant receptor such as T877A or W741L/W741C is present as seen in long-term ADT patients, many anti-androgens can agonize AR activity rather than antagonize it. In the case of over-expressed AR, experiments showed differential recruitment of co-activators, which are associated with agonist activity (97, 98). In LNCaP cells with the AR-activating mutation T868A, Bicalutamide did not show agonist properties to the mutant AR like other anti-androgen drugs (99). Bicalutamide is the commonly employed anti-androgen drug used in combination with LHRH agonists and antagonists in ADT therapy for treatment of patients with metastatic prostate cancer (94, 98). Although it was utilized in monotherapy and was initially lauded for its equal efficacy to castration without the side effects (100), Bicalutamide blocks AR activity in the pituitary and prostate, and LHRH release often ensues to increase the production of testosterone (98), so it is most effective in conjunction with LHRH inhibitors.

Enzalutamide (Xtandi; Astellas Pharma, US, Northbrook, I.L.) is in the next generation of nonsteroidal anti-androgens, and unlike drugs like Bicalutamide and Flutamide, it does not have agonist properties in the presence of overexpressed AR. Additionally, Enzalutamide also

blocks AR translocation (94). The Food and Drug Administration has now approved Enzalutamide to treat prostate cancer patients with metastatic disease who have completed treatement with the anti-mitotic drug Docetaxel (Sandoz; Princeton, NJ).

When a patient ceases to respond to one form of ADT, often a combination of orchiectomy and LHRH analogs/anti androgens are used, commonly referred to as combined androgen blockade (89). The primary side effects of ADT are decreased sex drive, menopausal symptoms, osteoporosis, and increased risk of hormone associated disease (86).

ADT-resistant patients can undergo treatments of chemotherapy, vaccines, and biophosphonates. In chemotherapy, drugs such Docetaxel and the DNA intercolator Doxorubicin (Pfizer; New York, N.Y.), to prevent cell division, are often employed. Side effects include hair loss, mouth sores, appetite suppression, nausea, and heart trouble. The vaccine Sipuleucel-T (Provenge; Dendrion Corrporation, Seattle, W.A.) entails the removal of white blood cells from the patient, exposure to prostatic acid phosphatase, and the intravenous delivery of the activated T-cells in a three part series to boost immune response to cancerous cells. Biophosphonates can be used to alleviate pain in patients with bone metastasis (89).

### 1.7.2 Novel Approaches in Prostate Cancer Treatments

There are currently several novel approaches to the treatment of prostate cancer. Some of these include novel AR-targeting drugs which target novel surfaces of the LBD (101) besides the area of helix 12 where ligand directly binds, as well as the N-terminal inhibitors which block transactivation of the receptor. Others include Hsp inhibitors which affect the stability of AR, histone deacetylase (HDAC) inhibitors which affect the transcription of AR and AR target genes, mammalian target of rapomycin (mTOR) inhibitors which prevent AR ligand-independent activation via the PI3K/Akt/mTOR pathway, and Src-kinase inhibitors which block signal

transduction of several cell surface receptors which are often active in CRPC patients (97). Two of these treatments are of particular interest to this project, the AR BF3-surface inhibitors and Hsp90 inhibitors.

***Androgen Receptor BF-3 Surface Inhibitors***

In 2007, the Fletterick group identified the BF3 surface on the AR ligand binding domain. Compound screenings of drugs that targeted this hydrophobic surface effectively prevented the binding of AR coactivators SRC2, SRC3, and ARA70 and resulted in decreased receptor signaling. X-ray diffraction images of the compounds Triac and flufenamic acid with AR revealed that rather than binding the coactivator binding surface AF-2 as initially expected, these compounds bound the BF-3 surface and altered the orientation of AF-2. The BF-3 cleft is located at the intersection of helix 9, the loop connecting helix 3 to helix 5, and helix 1. BF-3 surface inhibitors tremendously decrease receptor potentiation and represent a novel approach to receptor targeting as they may serve as an allosteric control to coactivator binding (101).

Our lab had characterizied many of the same BF-3 surface residues on AR that the Fletterick lab had targeted using their molecule. From our studies, this AR BF-3 surface was implicated as the putative binding site for the immunophilin and cochaperone protein FKBP52. Mutations to these residues were associated with a greater sensitivity to FKBP52 regulation of the receptor (102, 103). Mutations to BF3 surface residues are seen in patients with androgen insensitivity syndrome (104-107) and prostate cancer (105). Our lab developed a novel compound, MJC13, based on modifications of H7 parent compound, which is similar in structure to fenamic acid derivatives that the Fletterick lab developed. This drug binds to the AR LBD and likely binds the BF-3 surface of AR as predicted by *in –silico* docking simulations.

***Hsp90 Inhibitors***

For several years, scientists have theorized that Hsp90 could be a good therapeutic target for cancer treatments. Oncogenic proteins are overexpressed at such rapid rates that they become misfolded and unstable in cancerous conditions. These proteins are dependent on Hsp90 for functionality and existence. Indeed, it has even been asserted that "cancer cells are addicted to Hsp90" because without them, the misfolded oncogenes would be degraded and the cancerous cell could not propagate. The majority of Hsp90 inhibitors bind at the N-terminus, and recently designed ones now bind to the C-terminus (29).

In 1999, 17-N-Allylamino-17-demethoxygeldanamycin (17AAG) was the first Hsp90 inhibitor to reach Phase III clinical trials (108). Over 23 Hsp90 N-terminal inhibitors are in clinical trials presently (108). Hsp90 inhibitors seem to accrue more rapidly in cancer cells over non-cancerous cells (109, 110), and the inhibitors appear to be more potent towards cancer cells (111). Hsp90 has been used in conjunction with other drugs to help increase their efficacy. For example, 17-AAG was used effectively with Trastuzumab (Herceptin; Genentech, San Francisco, CA) in Phase II Human Epidermal Growth Factor Receptor 2 (HER2) + breast cancer clinical trials to the benefit of 57% of the patients (112). During Phase I and II trials of multiple myeloma, 17AAG was used with the proteasome inhibitor Bortezomib (Velcade; Millenium Pharmaceuticals, Cambridge, M.A.), and resistance to bortezomib was in some cases, overcome (113). This combinatorial approach actually induced apoptosis, and it is postulated that this happened because protein degradation was blocked and misfolded proteins were accumulating (114).

While Hsp90 inhibition was effective in treating several cancers in clinical trials, it was not effective in helping patients with CRPC. Cancer researchers originally thought that the

inhibitor would bring relief to CRPC patients since the androgen receptor is an Hsp90 client protein and the androgen receptor is often still active in castrate resistant prostate cancer. However, treatment with the Hsp90 inhibitor IPI-504 helped 25% of patients as evinced by a PSA decrease of nearly half, but in patients with bone metastasis, no change was seen (115). When 17AAG was used in another prostate cancer Phase II trial, there was no benefit to the patient (116).

Critics of these drugs have stated that while pro-cancer proteins are obvious targets of this therapy, other client proteins are also impacted. For example, Hsp90 inhibitor treatment in one experiment actually promoted transformation due to the loss of tumor suppressor Interferon Regulatory Factor 1 (IRF1). In the absence of IRF1 and the presence of a Ras mutation, transformation occurred (117). Additionally, the use of Hsp90 N-terminal inhibitors can prolong heat shock protein response. This is a problem in cancer treatment because Hsp90 disassembles transcriptionally active HSF-1 trimers. In a *Xenopus laevis* oocyte model, treatment with the Hsp90 inhibitor geldanamycin prolonged heat shock response (118). HSF-1 trimers actively promote oncogenesis (118, 119). Also, drug delivery and dosing must be carefully administered or results will be poor. In the case of one Phase II metastatic melanoma trial involving 17AAG, no benefit was shown to the patients, but further investigation of biopsies showed that Hsp90 was not effectively being inhibited (120).

Because of the heat shock response induced by N-terminal inhibitors, the novobiocin derived C-terminal Hsp90 inhibitors have been developed. One drug of promise is KU135. This drug was more effective in promoting apoptosis of Jurkat T cell leukemia lymphocytes than 17AAG (121).

Though these drugs administered alone are less effective at treating prostate cancer than other cancers, it is feasible that combinations of Hsp90 inhibitors and other drugs might increase their efficacy in a prostate cancer setting.

### 1.8 CURRENT RESEARCH FOCUS

In the present study, I demonstrate that β-catenin and FKBP52 synergistically potentiate AR signaling in the 52 KO MEF and 22RV1 prostate cancer cell lines. AR signaling increases thirty-fold over vector when β-catenin and FKBP52 are co-transfected with AR in a luciferase assay of 52 KO MEF cell lysates. β-catenin and FKBP52 can interact directly *in vitro* in the absence of other proteins, as demonstrated by a GST-pull down assay. As assessed in 52 KO MEFs, an FKBP52 K354A mutation, which disrupts binding to Hsp90, does not abrogate β-catenin and FKBP52 synergism of AR. FKBP52 does not require PPIase activity to produce AR synergism with β-catenin. However, FKBP52's proline-rich loop overhanging the PPIase pocket of FKBP52 is a critical structural determinant of synergism. Synergism is DNA sequence-specific, indicating that the impacts of synergism occur at the transcriptional level. The AR-specific inhibitor MJC13 effectively abrogates synergism with a half maximal inhibitory concentration ($IC_{50}$) of 1 μM in the 52 KO MEF model. MJC13 inhibition of receptor is not enhanced with the addition of Bicalutamide or the Hsp90 inhibitors KU174 and 17AAG. In studies comparing FKBP52 knock down 22RV1 (52 KD 22RV1) cells against wt 22RV1 control cells, β-catenin transfection only doubles AR transcriptional activity in the presence of minimal FKBP52. However, when FKPB52 is present as in the control cells, β-catenin transfection increases AR transfection activity one hundred fold over vector. This proves the importance of FKBP52 as a requirement for robust enhancement of AR signaling due to β-catenin.

## 1.9 HYPOTHESIS

FKBP52 and β-catenin interact directly in a synergistic manner to influence transcriptional events of the androgen receptor.

## 1.10 DISSERTATION GOALS

The overall goals of this work are to highlight the previously undocumented interactions of β-catenin and FKBP52 as they synergistically regulate AR in the 52 KO MEF model system, to block the synergism, and then to determine the relevance of this synergism in a prostate cancer cell line. An attempt has been made to understand this regulation of AR by addressing the following questions:

1. What are the important components of β-catenin and FKBP52 synergism?
2. Can we prevent FKBP52/ β-catenin synergism with inhibitors such as AR BF3 surface inhibitors, androgen antagonists, Hsp90 inhibitors, or combinations thereof?
3. Does FKBP52/ β-catenin synergism exist in prostate cancer cells?

In addressing these questions, we will elucidate some of the key elements by which the AR regulatory proteins β-catenin and FKBP52 work in tandem to coactivate transcription, and by therapeutically targeting this interaction, we offer an alternative method to prevent progression of prostate cancer. In testing our hypothesis, we not only gain a more complete understanding of the androgen receptor complex, but we present a novel therapeutic target for prostate cancer. The FKBP52/ β-catenin /SHR complex may be an effective target exploitable in treatment of other pathologies involving steroid hormone receptors.

# Chapter 2: Elucidation of the Important Components of β-catenin and FKBP52 Synergism

## 2.1 RATIONALE

Prior to the studies conducted here, β-catenin and FKBP52 had been studied separately as positive regulators of androgen receptor complex signaling pathways (11, 57, 81, 82, 122), but no studies have suggested that the proteins work in concert to regulate androgen receptor signaling. Initially, our studies in yeast suggested that AR BF3 surface mutations cause FKBP52 dependency (103), fenamic acid inhibitors which contact the AR BF3 surface at those same residues implicated for FKBP52 potentiation of AR prevent full AR signaling (101, 103), and because FKBP52 differentially regulated the steroid hormone family while in the presence of Hsp90 suggested direct contact of FKBP52 to AR (11-13), we believed that FKBP52 contacted AR at the BF3 surface. The Fletterick lab was analyzing data which showed that the BF3 surface of Liver Receptor Homologue-1 (LRH-1) was implicated as the binding site for β-catenin (123), and it was possible that β-catenin regulated the BF3 surface of AR, as well. No crystal structures had been generated for AR complexed with either β-catenin or FKBP52. Thus, we wished to investigate whether FKBP52 and β-catenin could compete for binding or work in tandem to regulate AR through the BF3 surface.

*Our initial hypothesis was that FKBP52 and β-catenin would compete for binding to the AR BF3 surface, and that co-transfection of both proteins with AR would result in a masking effect by which the stronger binding protein would mask the effect of the other*. To investigate this hypothesis, we would initially perform an AR-mediated reporter assay in mammalian cells to discriminate differences in AR reporter gene expression when FKBP52 and β-catenin were co-transfected (both individually and together) with AR and reporters. Following this initial assay, a pull-down assay was performed with FKBP52 and β-catenin, mutations were performed in

FKBP52 to characterize the components of regulation, and different AR promoters were utilized in AR mediated reporter assays to determine important components of regulation.

## 2.2 Materials and Methods

### 2.2.1 X-Ray Crystallography and Superposition of AR LBD over LRH-1 LBD

The crystal structure of β-catenin armadillo repeats (residues 138-663) complexed with LRH-1 hinge region (residues 191-541) was solved by the Fletterick laboratory as referenced (123). Briefly, fragments of β-catenin were subcloned into pCDF-2 Ek (EMD Millipore, Madison, WI) and fragments of LRH-1 were subcloned into pET32 Xa/LIC (EMD Millipore, Madison, WI), and the plasmids were transformed and grown into BL21Star (DE3) cells (Invitrogen, Carlsbad, CA). Batch purification was performed using Ni-nitrilotriacetate beads (Qiagen, USA) and secondary purification was performed with Superdex 200 10/30 (GE Healthcare, Sweeden). Proteins were concentrated, crystallized in 20mM Tris HCl (pH8.5) with 40% PEG200. Diffraction data was obtained at the Lawrence Berkeley National Laboratory (Beamline 8.3.1) (UCSF, CA), and molecular replacement was performed using Phaser in Collaborative Computation Project 4 Suite (CCP4) (Warrington, UK).

Superposition of the AR ligand binding domain with a BF3 binding molecule (Protein database 2PIT) over LRH-1 hinge LBD was performed with Topp in CCP4. The crystal structures and space fill models were generated from this superposition using CCP4.

### 2.2.2 Gal-4tk-Luc Assays in HeLa Cells

HeLa cells were maintained 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 (HyClone, Logan, UT) 24 hours prior

to the transfection. For transfections, cells were plated at 2.5 x$10^5$ cells/well for overnight incubation. 200 ng of the GAL4-responsive luciferase plasmid, 10 ng of β-actin β-galactosidase control plasmid, 10 ng of the GAL-AR expression vector or vector control (28), and 100 ng of β-catenin in pCI Neo (Addgene, Inc, Cambridge, MA) or empty pCI Neo vector control (Promega, Madison WI) were mixed with 0.5μL of Transfectin Lipid Transfection Reagent (Bio Rad, Hercules, CA) for 24 hours. Cells were collected and lysed in 100 μL of 100mM TrisHCl (pH7.5) with 0.1% Triton-X-100. Luciferase and β-gal were quantified using the luciferase assay system (Promega, Madison, WI) and the Gal Screen System (Applied Biosystems, Bedford, MA) according to manufacturers' specifications.

### 2.2.3 Receptor-Mediated Reporter Assays in 52 Knockout MEFs

For assays, immortalized 52 KO MEF cells, generated previously from FKBP52 knockout mice (13), 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 plated at a concentration of 2 x$10^6$ cells/well in 6-well plates at approximately 80% confluence. They were transfected in duplicates 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 either a pT81 (American Type Culture Collection, Manassas, VA) plasmid expressing firefly luciferase reporter driven by the androgen-dependent Probasin promoter, the androgen-responsive mouse mamory tumor virus (MMTV) promoter, or the PSA promoter. 800 ng of the pCI-Neo plasmid (Promega, Madison

WI) expressing human AR, and 800 ng of the pCI-Neo plasmid expressing FKBP52, FKBP51, FKBP51 or FKBP52 mutants, and/or 800 ng of pCMV-Sport6 plasmid (Open Biosystems, USA) expressing β-catenin, with 800 to 1600 ng of pCI-Neo plasmid containing normalizing vector. The PSA promoter and enhancer were previously subcloned into the pGL-2 basic (Promega, Madison, WI) firefly luciferase promoter; this plasmid was kindly donated by Marianne Sadar (124).

The S33A degradation resistant mutant form of β-catenin was constructed using forward and reverse primers as well as the Quick Change II Site Directed Mutagenesis Kit (Cell Signaling Technologies, USA) in a polymerase chain reaction (PCR) of the pCMV-Sport 6 β-catenin plasmid according to manufacturer's protocols. The FKBP51 and FKBP52 mutants were constructed using forward and reverse primers as well as the Quick Change II Site Directed Mutagenesis Kit (Cell Signaling Technologies, USA) in a PCR of the pCI-Neo plasmid expressing FKBP51 or FKBP52 according to manufacturer's protocols.

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 sixteen hours of incubation with hormone, cells in each well were lysed using 100μL mammalian protein extraction reagent (M-PER) (Pierce, Rockford, IL) supplemented with Complete ethylenediaminetetraacetic acid (EDTA)-free Mini Protease Inhibitor (Roche, Manheim, Germany) and spun using a 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 (incubation time was

approximately 5 minutes for the luciferase assay and 2 hours for the β-galactosidase assay), 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 SDs [error bars]) averaged. The data are plotted as the fold induction of AR in the presence of FKBP52 and β-catenin co-expression, as compared to AR activity in the presence of empty vector alone.

All data was represented as the mean (+/- standard deviation) of at least two separate samples, and figures are composite graphs representing data from at least three independent experiments.

### 2.2.4 Western Blot of Cellular Lysates

To confirm equivalent expression of each protein, Western immunoblots were performed using the prepared lysates from assays as described above. Duplicate samples were combined. After quantifying protein concentrations of each lysate, an equivalent amount of protein was loaded for each sample lane on Criterion gels (BioRad, Hercules, CA), and proteins were separated by electrophoresis. The proteins were then transferred from the gels to Immobilon Polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, ME), and the membranes were blotted for protein expression. The following mouse monoclonal antibodies were used as primary antibodies: anti-FKBP51 FF1 (epitope in FK1 domain), anti-FKBP52 Hi52D (epitope in FK1 domain), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Biodesign

International, Saco, ME) as a loading control. The following rabbit polyclonal antibodies were used as primary antibodies: anti-AR (N-20 Santa Cruz, Santa Cruz, CA) and anti-β-catenin (AbCam, Cambridge, MA). Anti-rabbit and anti-mouse secondary antibodies (Southern Biotechnology, Birmingham, AL) were used, and the ImmunStar Alkaline Phosphatase Substrate (BioRad, Hercules, CA) was applied prior to exposing to X-ray films for development and detection of antibodies.

### 2.2.5 *In vitro* Binding Studies

For the assessment of the ability of β-catenin to bind FKBP52 *in vitro*, a GST pull-down assay was performed. 20 µL Glutathione-Sepharose 4B (GE Healthcare, Sweeden) beads were added to microcentrifuge tubes, samples were centrifuged at at 4°C, and supernatant was removed. Beads were washed in ice-cold Binding Buffer [50mM HEPES (pH 7.4), 50mM KCl, 10mM $MgCl_2$, 0.01% Tween 20, 1 mM DTT] supplemented with EDTA free, Halt™ protease inhibitor cocktail (Pierce, Rockford, IL) and resuspended in 400µL of the Binding Buffer. GST-Tagged β-catenin (Millipore, Tamecula, CA) was added to two microcentrifuge tubes, and tubes were inverted using the Hula Mixer (Invitrogen, Oslo, Norway) for 1.5 hours at 4°C. All tubes were centrifuged at 4°C and supernatant was removed. Next, ice-cold Wash Buffer [50mM HEPES (pH 7.4), 50mM KCl, 10mM $MgCl_2$, 0.08% Tween 20, 1 mM DTT] supplemented with EDTA free, Halt™ protease inhibitor cocktail (Pierce, Rockford, IL) was employed for three washes. Samples were resuspended in 400µL Binding Buffer. An equimolar amount of bacterial batch-purified FKPB52 protein as β-catenin was added to two tubes: one containing GST with β-catenin and one with solely GST. All samples were inverted using the Hula Mixer (Invitrogen) for 1.5 hours at 4°C. Samples were centrifuged at 4°C and supernatant was removed. Samples were washed in Wash Buffer three times, and supernatant was removed. 15

μL of BME/4xSDS solution were added to each sample, including input samples of FKBP52 and β-catenin protein, and all samples were placed the heat block to denature at 95°C for five minutes. Samples were loaded onto Criterion gels (BioRad, Hercules, CA), and proteins separated by electrophoresis. The proteins were transferred from the gels to Immobilon PVDF membranes (Millipore, Bedford, ME), and the membranes were blotted for protein expression. The following primary antibodies were used to detect proteins of interest: Rabbit polyclonal to β-catenin (Millipore, Tamecula, CA) and mouse monoclonal anti-FKBP52 Hi52d (epitope in FK1 domain). Anti-rabbit and anti-mouse secondary antibodies (Southern Biotechnology, Birmingham, AL) were used in detection of primary antibodies. The ImmunStar Alkaline Phosphatase Substrate (BioRad, Hercules, CA) was applied prior to exposing to X-ray films for development and detection of antibodies.

## 2.3 RESULTS

### 2.3.1 Identification of a Putative Interaction Surface between the Region Surrounding AR's AF-2 Surface and the β-catenin Armadillo Repeats

An X-ray crystallographic structure of the β-catenin armadillo repeats complexed with the LRH-1 hinge LBD suggests interaction between the third through seventh armadillo repeats of β-catenin and a region encompassing the AF-2 and BF-3 surfaces of the receptor's ligand binding domain (123). Mammalian-2-hybrid studies have suggested that β-catenin binds the androgen receptor similarly near its AF-2 surface (81). Other studies employing yeast two-hybrid studies (82) as well as GST pull-down assays (85) have shown that β-catenin's armadillo repeats are crucial for interactions with AR. GST-pull-down assays revealed that β-catenin mutants Y306A, K345A, and W383A do not bind to LRH-1 or AR well. In these studies,

binding to AR or LRH-1 was only possible with the wild-type β-catenin protein; the study suggests that β-catenin interacts AR similarly to its association with LRH-1 (123). These results established commonalities between the LRH-1 and AR interfaces with β-catenin.

**Figure 2.1** presents the superposition of the interaction of β-catenin armadillo repeats with the LRH-1 hinge LBD (Protein Database 3TX1) superposed with the AR LBD with the [4-(4-hydroxy-3-iodo-phenoxy)-3,5-diiodo-phenyl]-acetic acid molecule bound (Protein Database 2PIT). The LRH-1/ β-catenin interaction appears in green; the AR LBD appears in light blue, and the fenamic acid molecule, which highlights to BF3 surface, appears in dark blue. Superposition was generated using the Topp program in CCP4. This figure shows similarity of domain orientations between the ligand binding domains of the two receptors at their interface with β-catenin.

### 2.3.2 FKPB52/β-catenin Effects on AR Potentiation

The crystal structure and superposition study shown in **Figure 2.1** presented data which warranted investigation of β-catenin's regulation of AR, and it was important to compare this with the regulation of AR by FKPB52, which we also believed to be mediated through AR's BF3 surface. To determine if FKBP52 and β-catenin would function competitively or in concert in regulation of AR, luciferase assays were conducted in mammalian expression systems. We investigated if the ligand binding domain of the androgen receptor would be differentially regulated by the proteins FKBP52 and β-catenin when the proteins were introduced individually versus together in the HeLa cell line. Because wild-type β-catenin is rapidly phosphorylated by GSK3β to undergo degradation when it is over-expressed in a cell (70), we utilized the β-catenin S33Y mutant, which is resistant to GSK3β-mediated phosphorylation.

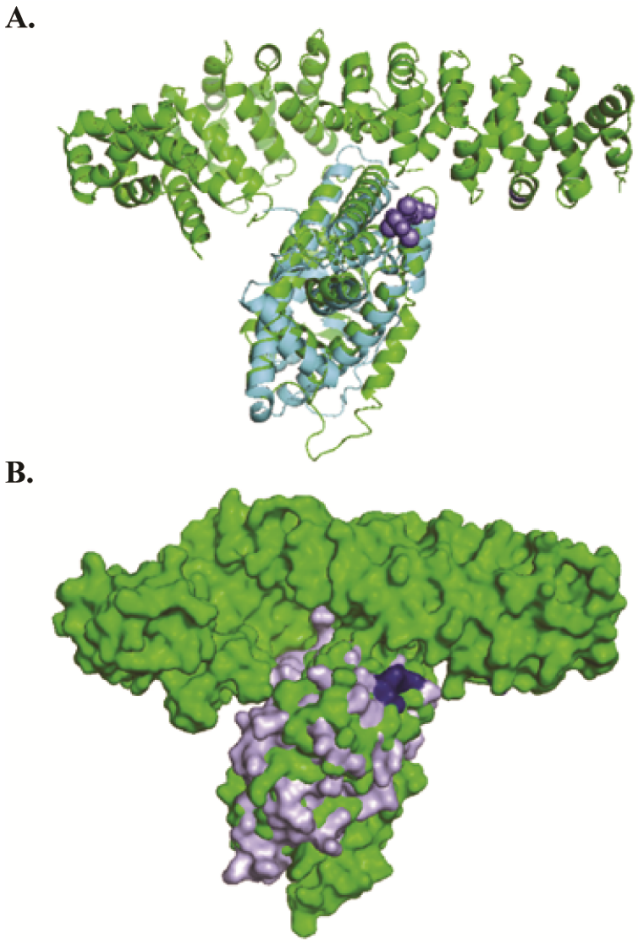



Figure 2.1: β-catenin Binds a Surface of LRH-1 Ligand Binding Domain That Overlaps with the Putative FKBP52 Interaction Surface.

A. The AR LBD crystal structure (cyan) bound to a BF3 binding molecule (purple) overlaid on the LRH-1/ β-catenin (green) co-crystal structure. Note that the predicted β-catenin binding site on AR overlaps with the putative FKBP52 interaction site (BF-3 surface).

B. Space-filling model of LRH-1/ β-catenin (green) and AR BF-3 (gray).

A mammalian vector expressing the AR LBD fused to the Gal4 DNA binding domain was employed in co-transfection assays of HeLa cells with the Gal4-tk-luc reporter. β-catenin and FKBP52 were co-transfected alone and together, in the presence of ethanol or DHT, and a vector was used as a transfection control. In the absence of DHT, none of the transfected groups signaled, verifying that the Gal4-AR LBD was responsive to DHT treatment. In the presence of DHT, induction was influenced greatly by the transfected plasmids. The Gal4/AR LBD increased Gal4-dependent transcription appropriately. When β-catenin was transfected alone, it increased transcription eight-fold. Transcription doubled when FKBP52 was transfected. Instead of exhibiting competition, when the two proteins were transfected together, activity was synergistic; transcription increased fifteen-fold over vector alone (**Figure 2.2A**).

An ideal model system would provide a negative background for studying the proteins FKBP52 and β-catenin. Our lab possessed a mouse embryonic fibroblast cell line generated from FKBP52 knockout mice (11). Because MEFs are a hearty, stable cell line lacking FKBP52 and AR, and MEFs express FKBP51 nearly undetectable levels, this line was utilized for the initial characterization of these proteins. Mammalian expression plasmids were transiently transfected into 52 KO MEF cells to determine the effects of co-transfecting β-catenin alone, FKBP52 alone, and β-catenin with FKBP52, along with the AR-inducible Probasin luciferase reporter plasmid and a β-galactosidase reporter plasmid to normalize for transfection efficiency. Following a 16-hour hormone induction period with 10 pM DHT or vehicle control to dissect hormone-specific effects, cells were lysed, and AR-mediated reporter assays were conducted. Following normalization for transfection efficiency, reporter expression was quantified as a percentage over maximum signaling for each of the following treatment groups: vector, FKBP52, β-catenin, FKBP52 + β-catenin, β-catenin S33A, and FKBP52 + β-catenin S33A.

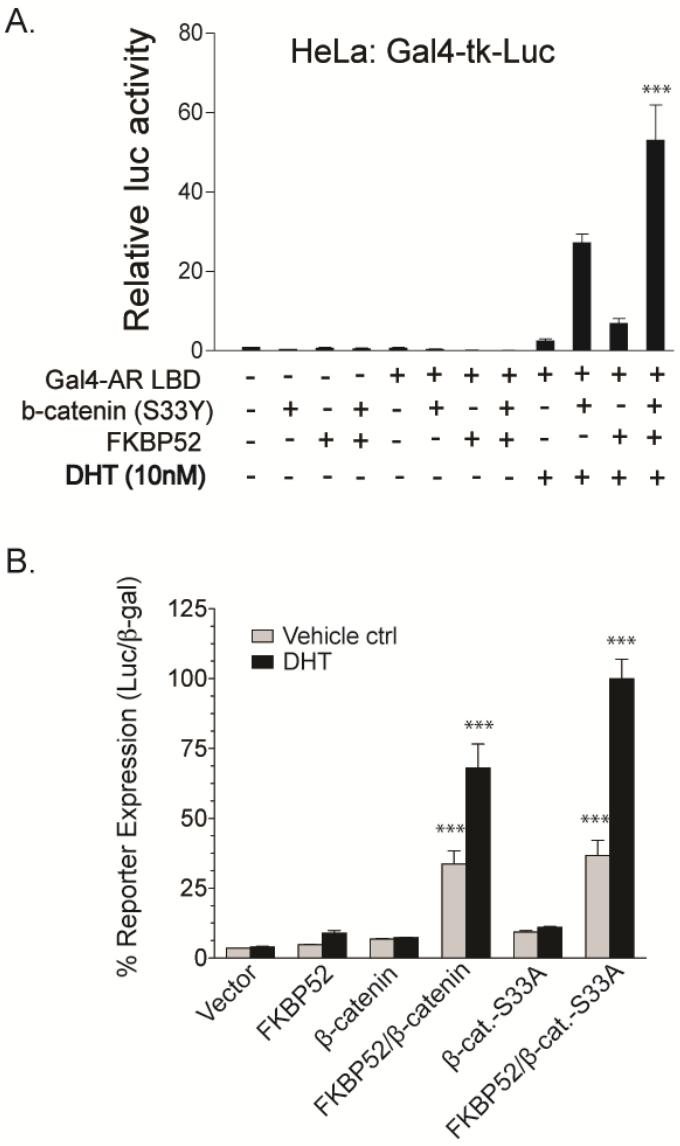



Figure 2.2: FKBP52 and β-catenin Synergize in HeLa and 52KO MEF Cells.

Figure 2.2: FKBP52 and β-catenin Synergize in HeLa and 52KO MEF Cells.

A. Reporter assays were performed in HeLa cells using the Gal4-tk-Luc reporter system in the presence or absence of Gal4-AR LBD, β-Catenin, FKBP52, or hormone (DHT). The data represent averages (plus SDs [error bars]) of at least three independent experiments. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, Gal4-AR LBD luc reporter activity is significantly enhanced in cells transfected with FKBP52/ S33Y β-Catenin as compared to those transfected with either FKBP52 or S33Y β-Catenin when comparing cells enhanced with DHT, with a p-value < 0.001 (***).

B. Wild type AR, the AR-inducible luciferase reporter plasmid, and the constitutively active β-galactosidase reporter plasmid were co-transfected simultaneously into 52KO MEFs with each of the plasmids indicated for the different treatment groups. Cells were induced with 10 pM DHT (black bars) or ethanol (gray bars). Following cell lysis, AR transcriptional activity was tested through a luciferase assay, followed by a normalizing β-galactosidase assay. Bars represent luciferase reporter expression/ β-galactosidase reporter expression and are averages (plus SDs [error bars]) of at least three independent experiments. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, AR reporter activity is significantly enhanced in cells transfected with FKBP52/ wild typeβ-Catenin as compared to those transfected with either FKBP52 or wild type β-Catenin when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value < 0.001 (***). AR reporter activity is also significantly enhanced in cells transfected with FKBP52/ S33A β-Catenin as compared to those transfected with either FKBP52 or S33A β-Catenin when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value < 0.001 (***).

FKBP52 doubled AR activity as compared with vector in the presence of DHT. β-catenin slightly enhanced activity in the presence or absence of hormone, and that regulation was further enhanced when the GSK3β phosphorylation deficient mutant, β-catenin S33A, was utilized. Synergism was clearly seen through a thirteen-fold induction over vector signaling when β-catenin and FKBP52 were co-transfected in the presence of DHT. Without DHT, the induction was approximately six-fold whether using wild type or S33A β-catenin. Synergism is maximized to twenty-fold when the S33A form of β-catenin is used in the presence of DHT, as would be expected (**Figure 2.2B**).

### 2.3.3 β-catenin and FKBP52 Proteins Interact Directly *in vitro*

It is currently understood that FKBP52 must be bound to the AR complex to exert its effects on AR-mediated transcription. Steroid hormone receptors bind Hsp90, and Hsp90 binds FKBP52 (125, 126). The Hsp90 inhibitor geldanamycin prevents association of FKPB52 with AR in *in-vitro* interactions employing co-IP reactions with rabbit reticulocyte lysate (11). A K354A mutation in the TPR region of FKBP52 prevents the binding of FKBP52 to Hsp90. This mutation is able to prevent regulation of GR by FKBP52 (12). However, it was an undeniable possibility that we had discovered a new mechanism of regulation of AR by FKBP52 that operated independently of any other proteins.

To determine if β-catenin and FKBP52 could interact in the absence of the androgen receptor complex or other proteins, GST pull-down assays were performed using GST-tagged β-catenin and purified FKBP52 proteins. FKBP52 did not significantly bind the beads in the absence of β-catenin, yet GST-tagged β-catenin did bind the beads, indicating that the interaction was specific. An approximation was made to add equimolar levels of starting material, as indicated by the input visualized on the blot. GST-tagged, purified β-catenin binds FKBP52

directly in the absence of other proteins, as assessed by the Western blot of glutathione-sepharose bound beads (**Figure 2.3**).

In addition to the interaction studies discussed above, our collaborators at the Methodist Hospital in Houston (Dr. Paul Webb's group) have performed a number of co-immunoprecipitations. Their preliminary data validates our findings of a direct interaction between FKBP52 and β-catenin and shows clear evidence of a heteromeric complex containing FKBP52, β-catenin, and AR LBD.

### 2.3.4 Characterization of FKBP52's Structural Components for β-catenin Synergism

Since β-catenin and FKBP52 can interact *in vitro* in the absence of other proteins, we surmised that regulation of AR by these proteins could operate independently of what has previously been characterized in FKBP52 studies. It was important to determine the functional effects of FKBP52 muations on FKBP52/β-catenin synergism. Initial studies of FKBP52's regulation of steroid hormone receptors have shown that FKBP52's regulation is dependent on structural integrity of the proline-rich loop over the PPIase domain (12, 45). The mutants F130Y, F67Y, W90L, and Y57A abrogate PPIase activity without disrupting the conformation of the proline-rich loop or inhibiting FKBP52 regulation of AR (45). We wished to determine if PPIase activity was necessary for FKBP52/β-catenin synergism. Therefore, site-directed mutagenesis was performed to generate the F130Y and F67Y mutations on FKBP52 in a mammalian expression plasmid. Effects of these mutations were examined in our 52KO MEF system. Wild type AR, the AR-inducible Probasin reporter luciferase plasmid, and the β-galactosidase reporter plasmid (for normalizing transfection efficiency) were transiently transfected simultaneously with each of the plasmids indicated for the different treatment groups into 52 KO MEF cells. Cells were induced with 10 pM DHT (black bars) or vehicle control (gray bars).

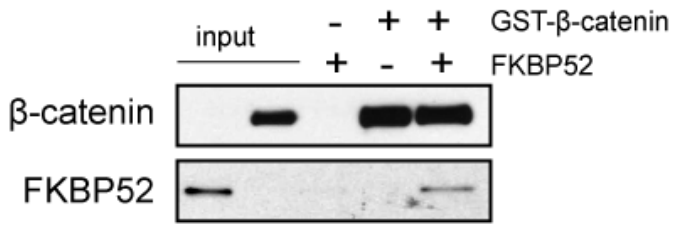



Figure 2.3: FKBP52 and β-catenin Interact in GST Pull-down Assay.

*In-vitro* GST-pull down assays were performed with purified, recombinant FKBP52 and GST-Tagged, purified β-catenin. Proteins were visualized with primary antibodies specific to human FKBP52 and β-catenin.

Following 16-hour incubation, cells were lysed, AR transcriptional activity was tested through a luciferase assay, and transfection efficiency was normalized through a β-galactosidase assay. These results confirmed that PPIase activity is not required for β-catenin/FKBP52 synergism to occur (**Figure 2.4A**). Though only the FKBP52 F130Y mutant is shown, results were also replicated using the FKBP52 F67Y PPIase-deficient mutant.

Previous studies of the FKBP51 double mutant A116V/L119P demonstrated the importance of the overhanging loop of the PPIase domain in regulation of AR (45). This region was studied because the FD67DV mutation, which disrupted PPIase activity (but also distorted the conformation of the PPIase domain), completely prevented potentiation of GR and reduced AR potentiation as well (12), yet other mutations which disrupted PPIase activity had no effects on FKBP52 potentiation of receptor. The FKBP51 L119P/A116V mutant was generated as a result of information from random mutagenesis of FKBP52's closely related protein FKBP51, which conferred FKBP52-like potentiation of AR. The L119P mutation results in a change in conformation of the overhanging loop that makes FKBP51 more structurally similar to FKBP52 (45). Thus, these studies demonstrated that FKBP51 and FKBP52 are functionally divergent proteins by virtue of only a few residues in the proline-rich loop. To determine if β-catenin could synergize with FKBP51 or the gain-of-function mutant FKBP51 A116V/L119P, we transiently transfected these plasmids alongside β-catenin and FKBP52 as described above. Indeed, the FKBP51 double mutant synergized in the presence of DHT with β-catenin but not with wild type FKBP51 (**Figure 2.4B**). This establishes the importance of preserving structural integrity of the proline rich loop for FKBP52/ β-catenin synergism.

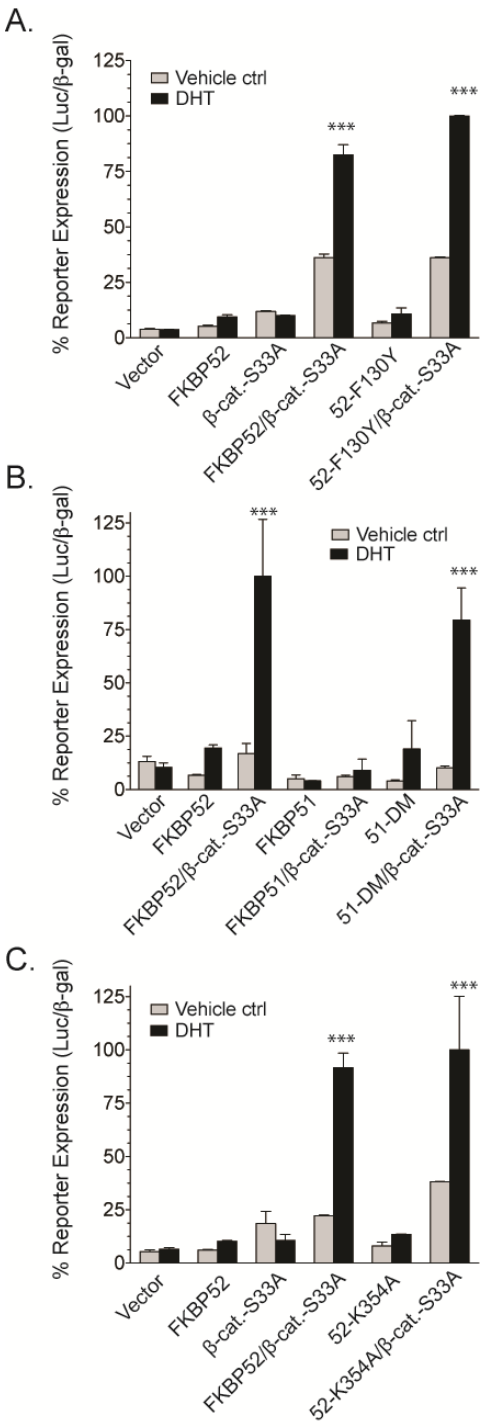



Figure 2.4: The Domain Requirements for FKBP52/β-catenin Synergy Mirror Those of FKBP52–Mediated Potentiation of Receptor Function

Figure 2.4: The Domain Requirements for FKBP52/β-catenin Synergy Mirror Those of FKBP52–Mediated Potentiation of Receptor Function

Wild type AR, the AR-inducible luciferase reporter plasmid, and the constitutively active β-galactosidase reporter plasmids were co-transfected simultaneously with each of the plasmids indicated for the different treatment groups. Cells were induced with 10 pM DHT (black bars) or ethanol (gray bars). Following cell lysis, AR transcriptional activity was tested through a luciferase assay, followed by a normalizing β-galactosidase assay. The data shown are averages (plus SDs [error bars]) of four independent experiments.

A. The PPIase-deficient mutant FKBP52-F130Y is compared to wild type FKBP52 for receptor potentiation. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, AR reporter activity is significantly enhanced in cells transfected with FKBP52-F130Y / S33A β-Catenin as compared to those transfected with either FKBP52-F130Y or S33A β-Catenin when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value $< 0.001$ (***). The already established wild type FKBP52/ S33A β-Catenin group is also statistically significant at $p < 0.001$ (***). There is no statistical significance between transfections using wild type FKBP52/ S33A β-Catenin as compared to F130Y FKBP52/ S33A β-Catenin when vector is utilized, but there is a statistical significance at ($p < 0.001$ (***)) when hormone is added to those transfected groups.

B. DM refers to the A116V /L119P double mutation in the FKBP51 proline-rich loop. This gain-of-function mutant allows FKBP51 to potentiate receptor similarly to FKBP52 was compared against wild type FKPB52 for receptor potentiation. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, AR reporter activity is

significantly enhanced in cells transfected with FKBP51-A116V /L119P / S33A β-Catenin as compared to those transfected with either FKBP51-A116V /L119P or S33A β-Catenin when comparing cells enhanced with DHT or treated with vector control for each perspective group, with a p-value < 0.001 (***). The already established FKBP52/ S33A β-Catenin group is also statistically significant at p < 0.001 (***). There is no statistical significance between transfections using FKBP52/ S33A β-Catenin as compared to FKBP51-A116V /L119P / S33A β-Catenin when vector is utilized, but there is a statistical significance at (p < 0.001 (***)) when hormone is added to those transfected groups.

C. The Hsp90 deficient-binding mutant FKBP52-K354A was compared against wild-type FKBP52 for receptor potentiation. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, AR reporter activity is significantly enhanced in cells transfected with FKBP52-K354A / S33A β-Catenin as compared to those transfected with either FKBP52-K354A or S33A β-Catenin when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value < 0.001 (***). The already established FKBP52/ S33A β-Catenin group is also statistically significant at p < 0.001 (***). There is no statistical significance between transfections using FKBP52/ S33A β-Catenin as compared to FKBP52-K354A / S33A β-Catenin when vector or DHT are utilized.

Since synergism with the FKBP51 double mutant was only 80% of the synergism seen with FKBP52, it appears that other functional determinants present in FKBP52 besides the overhanging loop that are absent in FKBP51 are important for full synergism with β-catenin.

Hsp90 chaperoning of the mature receptor complex is important for FKBP52's regulation of the receptor. A K354A mutation to the TPR domain of FKBP52 completely prevents its association with Hsp90 (12, 127). This mutation tremendously decreases GR potentiation by FKBP52 in a yeast model system (12). Because our earlier findings suggested that β-catenin can bind FKBP52 in the absence of Hsp90, we wanted to determine the functional effects of the FKBP52 K354A mutation on FKBP52/β-catenin synergism. Plasmids were transfected and assays performed as previously described. In the 52 KO MEF model system, we did not see any changes in AR potentiation due to a K354A mutation on FKBP52's regulation of AR alone or in conjunction with β-catenin (**Figure 2.4C**). Thus, coregulation of AR by FKBP52 and β-catenin does not require FKBP52 binding to Hsp90.

### 2.3.5 FKBP52/ β-catenin Synergism Is Reporter-Specific

Though FKBP52 is traditionally viewed as a cytosolic protein, it also associates with nuclear importins (3, 21) and the cytoplasmic motor protein dynein (22, 128). β-catenin is generally complexed with the membrane-bound E-cadherin protein, but during wnt-induction or in cancer pathways, β-catenin localizes to the nucleus (54). FKBP52/ β-catenin interactions could therefore occur in the nucleus or in the cytosol and could impact AR signaling at a number of regulatory points. To determine if FKBP52/ β-catenin synergism could enhance different promoters to varying degrees, the AR-responsive promoters MMTV (mouse mammary tumor virus), PSA (prostate specific antigen), and Probasin were examined in the context of luciferase reporter constructs. Probasin is a promoter found in mice which shows AR-specific and tissue-

specific regulation and is employed to study advanced prostate cancer (129). The MMTV promoter was initially characterized in mammalian cells and contains an HRE, which is inducible by GR, AR, and PR (130, 131). PSA is a promoter that drives the serum biomarker for prostate cancer, although this gene is also transcribed at lower levels in normal prostate epithelia (132). Assays used in Figures 2.2b and 2.4 were generated using the Probasin promoter.

Experiments were conducted to determine DNA-specific effects on AR-mediated transcription. 52 KO MEF cells were cotransfected with one of the three androgen-responsive luciferase plasmids, the β-galactosidase reporter plasmid for transfection normalization, and the androgen receptor. DHT dose-response curves were generated for cells under the influence of each promoter, and the $EC_{20}$ was used for studies to follow. Initially, transfection cocktails for each promoter experiment included the reporter plasmids, AR, and plasmids for the following: vector, FKBP52, β-catenin S33A, or cotransfection of β-catenin S33A and FKBP52. Reporter gene expression was induced with minimal DHT for 16 hours. Following cell lysis, transcriptional activity was assayed through luciferase assays normalized by β-galactosidase expression. The individual graphs generated by each promoter are seen in **Figures 2.5A, 2.5B, and 2.5C.** Interestingly, in these studies, FKBP52 co-transfection with reporters and AR increased AR activity twofold over vector, regardless of the promoter. β-catenin co-transfection had negligible effects, as seen on all promoters. However, great differences in synergism were seen; Probasin showed the highest degree of synergism at 24-fold over vector but MMTV and PSA only showed slight increases in effect when β-catenin S33A was added with FKBP52. To generate the graph seen in **Figure 2.5D**, the AR promoter-specific luciferase reporter expression in the presence of FKBP52 and β-catenin was divided by AR activity in the presence of empty parent vector alone.

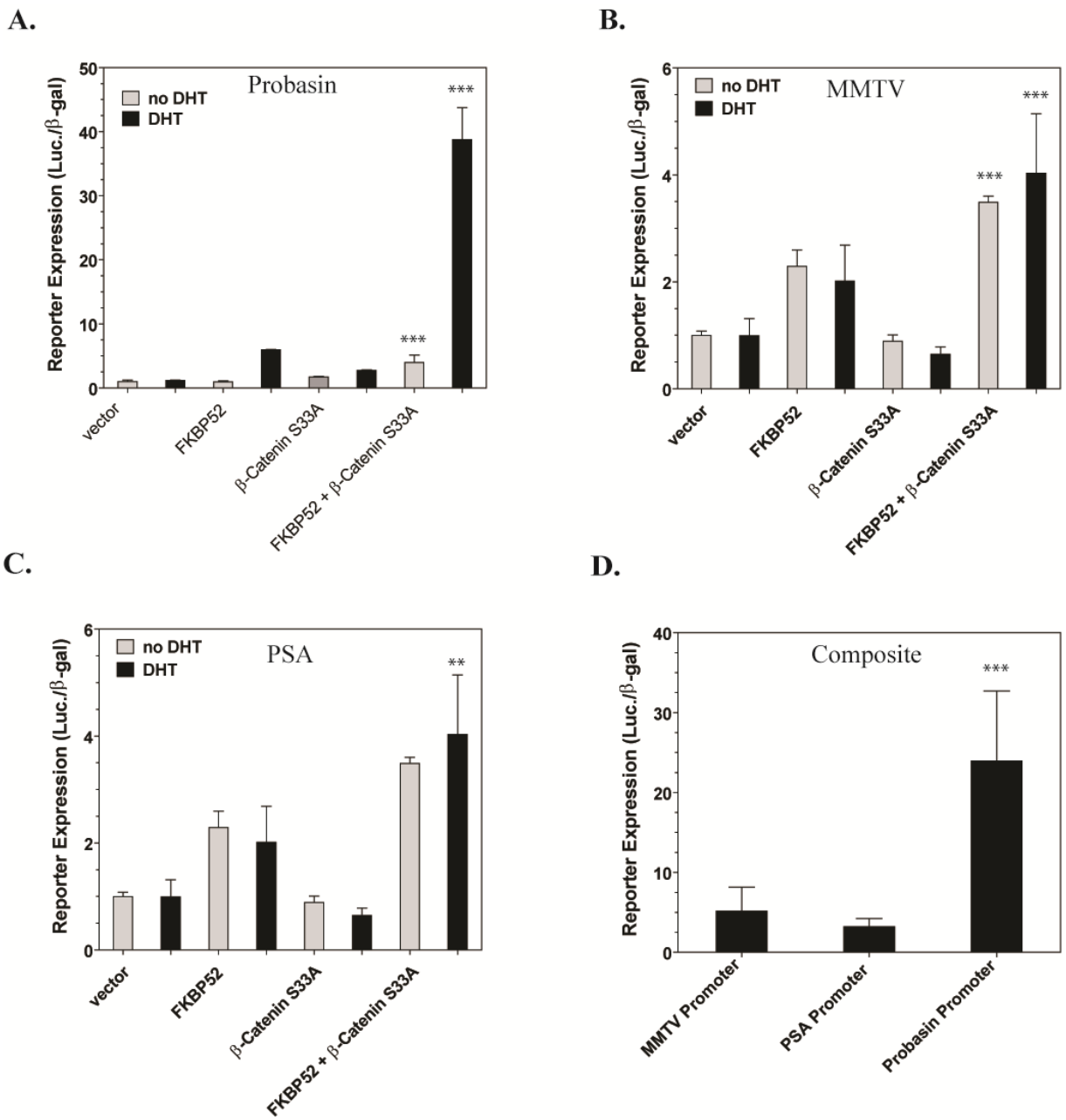



Figure 2.5: FKBP52/β-catenin Synergism Is Reporter-Specific.

Figure 2.5: FKBP52/β-catenin Synergism Is Reporter-Specific.

Wild type AR, the AR-inducible luciferase reporter plasmid driven by the Probasin (A), MMTV (B), and PSA (C) promoters, and the constitutively active β-galactosidase reporter plasmids were co-transfected simultaneously with vector, FKBP52, β-Catenin, or FKBP52 and β-catenin into 52KO MEF Cells. Cells were induced with 10 pM DHT (black bars) or ethanol (gray bars). Following cell lysis, AR transcriptional activity was tested through a luciferase assay, followed by a normalizing β-galactosidase assay. The data shown in A, B, and C represent five independent experiments (plus SDs [error bars]) averaged. The data shown in D is plotted as the fold induction of averaged AR activity in the presence of DHT for a given promoter in the presence of FKBP52 and β-catenin co-expression, as compared to AR activity in the presence of empty vector alone.

A) Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, Probasin reporter activity is significantly enhanced in cells transfected with FKBP52/ S33A β-Catenin as compared to those transfected with either FKBP52 or S33A β-Catenin alone when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value $< 0.001$ (***).

B) Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, MMTV reporter activity is significantly enhanced in cells transfected with FKBP52/ S33A β-Catenin as compared to those transfected with solely S33A β-Catenin when comparing cells enhanced with DHT or treated with vector for each perspective group, with a p-value $< 0.001$ (***). In the DHT-treated cells transfected with FKBP52 versus cells transfected with FKBP52/ S33A β-Catenin, there was a significant difference with a p-value $< 0.01$ (**). There is no

statistical significance between transfections using FKBP52/ S33A β-Catenin as compared to FKBP52 transfection alone when only vector control is utilized.

C) Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, PSA reporter activity is significantly enhanced in cells transfected with FKBP52/ S33A β-Catenin as compared to those transfected with solely S33A β-Catenin when comparing cells enhanced with DHT with a p-value < 0.01 (**). There is no statistical significance between transfections of FKBP52/ S33A β-Catenin as compared to solely S33A β-Catenin when comparing cells enhanced with vector control. There is also no statistical significance between cells transfected with FKBP52/ S33A β-Catenin as compared to those transfected with solely FKBP52, either in the presence or absence of DHT.

D) Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, Probasin Reporter activity is significantly different from both PSA and MMTV promoter activity with a p-value < 0.001 (***).

The only reporter which showed dramatic synergy (up to twenty-four-fold) was the Probasin promoter. The other reporters exhibited three to fourfold enhancement of receptor activity over vector in response to FKBP52/ β-catenin S33A co-transfection. Indeed, the co-regulation of AR activity by FKBP52 and β-catenin S33A depends on the specific DNA sequences on which the receptor complex acts.

## 2.4 DISCUSSION

From the initial idea that β-catenin could potentially regulate the androgen receptor at the same BF3 surface on which FKBP52 showed the greatest effects (**Figure 2.1**), this project has evolved to feature a novel interaction which is previously undocumented in literature. Though the initial hypothesis that β-catenin and FKBP52 would compete for binding the BF3 surface of AR was disproven, Chapter 2 documents the characterization of synergism between the proteins, which is of far greater value to not only the steroid hormone receptor field, but also to reproductive cancers involving AR.

FKBP52 and β-catenin work in concert to promote synergistic effects on AR signaling (**Figure 2.2**). The mere fact that this synergism causes the FKBP52 effect on AR to appear nominal, demonstrates the depth of this discovery. More importantly, in the absence of FKBP52, β-catenin does not appear to greatly enhance AR signaling (**Figure 2.2B**). β-catenin is well documented as a major contributor to prostate cancer progression, and our studies show that the role of β-catenin in this pathway can only occur when FKBP52 is present. Previous studies of β-catenin and AR did not utilize cell lines which lacked FKBP52. Preliminary studies of our collaborators suggest that the β-catenin/AR interactions are strengthened by the presence of FKBP52. Our findings in GST pull-down experiments suggest that FKBP52 and β-catenin can bind in the absence of other proteins, including AR and Hsp90 (**Figure 2.3**). The synergism is

not produced as a result of FKBP52 PPIase activity (**Figure 2.4A**), nor is it dependent on Hsp90 (**Figure 2.4C**), but it is related to FKBP52's regulatory surface, the overhanging proline rich loop above the PPIase pocket (**Figure 2.4B**). Lastly, the DNA sequence-specific effects on synergism as seen in the reporter studies suggest that the FKBP52/β-catenin synergistic effect is occurring at the transcriptional level (**Figure 2.5**).

These findings present a novel role for FKBP52 in AR pathways, in which the protein is critical for β-catenin enhancement of AR activity. Further studies are needed to continue to characterize this interaction and to develop better drugs to target it in cancer therapies. Chapter 3 highlights an initial examination of novel combinations of treatments, and Chapter 4 illustrates the significance of our findings in prostate cancer cell lines.

# Chapter 3: Investigation of AR BF3 Surface Inhibitors, Androgen Antagonists, Hsp90 Inhibitors, or Combinations of Inhibitors in Prevention of FKBP52/β-catenin Synergism

## 3.1 RATIONALE

Our laboratory developed a small molecule inhibitor, MJC13, which effectively abrogates FKBP52's potentiation of AR in yeast, MEFs, MDA-kb2 cells, and the prostate cancer cell lines LNCaP, LAPC4, VCaP, and 22RV1 (103). This inhibitor is structurally similar to some of the fenamic acid inhibitors used by the UCSF Fletterick lab, which bind a novel regulatory surface, BF3, of the androgen receptor. The BF3 surface appears to affect the orientation of AF-2, thereby regulating the recruitment of coactivators. When Flettericks' inhibitors bound BF3, the interaction weakened coactivator binding affinity on AF-2 (101). Feasibly, MJC13 could operate similarly, but it contacts different residues than Fletterick's compounds on the BF3 surface as predicted by *in silico* docking simulations. Functionally, MJC13 shows different trends from Fletterick's flufenamic acid as MJC13 is specific in blocking the FKBP52 effect on AR (and GR) potentiation. With flufenamic acid, the effects of FKBP52 inhibition are not as dramatic; it inhibits the receptors with less specificity for the FKBP52 effect. Therefore, a new mechanism of action is suspected. Western blots reveal that as MJC13 increases in concentration, the concentration of AR and FKBP52 proteins increases in the cells, similarly to the use of nonhydrolyzable ATP analogues that block Hsp90 complex dissociation. Employment of cellular fractionation shows that hormone-induced translocation is indeed blocked in a dose-dependent manner by MJC13. Enzyme-linked immuno sorbent assays (ELISA) reveal that MJC13 decreases secretion of PSA in LNCaP and VCaP cells. Western blots show a decrease in the AR-dependent proteins FKBP51 and PSA as MJC13 concentrations increase. Quantitative PCR reveal that transcription of both PSA and Human Transmembrane protease serine 2 (TMPRSS2) are prevented by MJC13. In 22RV1 cells, however, MJC13 only affects the androgen-responsive receptor with an intact LBD but not the constitutively active AR of this cell

line. These trends suggest that MJC13 prevents AR complex dissociation, thereby freezing AR in the cytoplasm and blocking transcription of AR-regulated genes through interaction with the AR LBD (103).

17AAG is a benzoquinone ansamycin derivative of geldanamycin which directly binds Hsp90 at its N-terminus to inhibit ATP binding, thereby inhibiting Hsp90 and even leading to the direct degradation of some of its client proteins (133, 134). KU174 is a second-generation derivative of novobiocin, which binds Hsp90 at the C-terminus to inhibit its activity (135). KU174 was developed as an alternative Hsp90 inhibitor to 17AAG to avoid inducing HSF1 and initiating the chemoprotective heat shock response in cells (135-137). Profiles of client protein degradation both overlap and contrast in comparisons of 17AAG with KU174 (135).

Bicalutamide is a nonsteroidal toluidide, a classic androgen antagonist that binds the hormone binding pocket of AR to prevent binding of DHT (138, 139). Experiments have suggested multiple mechanisms of action for this drug. In one study, a co-immunoprecipitation (co-IP) with AR in LNCaP cells incubated for 30 minutes at 37°C with Bicalutamide pulled down Hsp90, Hsp70, and FKBP52, even when hormone was added. Additionally, no AR was found in nuclear extracts incubated with Bicalutamide and hormone (99). However, Masiello et al. suggested that AR's localization to the cytoplasm could be a temporary state with this drug. When injected every other day for two weeks into mice with normal prostate epithelium, Bicalutamide-treated mice exhibited the same expression of nuclear AR as PBS-treated mice. Human bone marrow biopsies of malignant prostate cancer patients treated with Bicalutamide displayed nuclear AR in immunostained tissues. Bicalutamide or DHT was next incubated with LNCaP cells grown on coverslips for two hours and fixed for immunofluorescence. In two hours' time, Bicalutamide induced nuclear translocation of AR just as efficiently as 10 nM DHT.

Chromatin immunoprecipitation (ChIP) assays in LNCaP and AR-negative DU145 prostate cancer cells showed that Bicalutamide stimulated endogenous AR binding to the PSA enhancer. To shed light on the role of this anti-androgen inhibitor, a mammalian-two-hybrid protein-binding assay showed that Bicalutamide prevented the AR N- and C-terminal interactions for transactivation. Also, SRC-1/SRC-2 transfection did not stimulate Bicalutamide enhancement of AR activity (140), implying that coactivator recruitment is affected by Bicalutamide.

What is fascinating is that Bicalutamide seems to stimulate apoptosis even in AR-negative cell lines. Floyd et al. studied the spectrum of prostatic cell lines, from the androgen-sensitive benign hyperplasia-derived PWR-1E cells to the malignant, AR-negative PC-3 and DU-145 cells, for mechanisms of apoptosis. In all lines, Bicalutamide induced cell death. In the PWR-1E cells, apoptosis occurred within 24 hours and was caspase-dependent but not calpain-dependent. For the PC-3 line, the mechanism was partially caspase dependent, partially calpain-dependent (141). Therefore, Bicalutamide's mechanisms of action may differ depending on the androgen receptor dependency of the cell line.

The chemical structures of the compounds MJC13, Bicalutamide, 17AAG, and KU174 are illustrated in **Figure 3.1.**

Three main approaches to counteract FKBP52/ β-catenin synergism on AR could be to block the FKBP52/Hsp90 interaction, to prevent AR BF3 interactions with proteins, and to abrogate the β-catenin/AR interaction. At the heart of all of these interactions is the AR protein. We chose to focus on the blocking of AR BF3 interactions with the drug MJC13. By adding in the two classes of drugs anti-androgens and Hsp90 inhibitors, we believed that we could dramatically decrease synergism. Specifically, Bicalutamide would block ligand from binding receptor and conversely, the Hsp90 Inhibitors 17AAG and KU174 would prevent the receptor

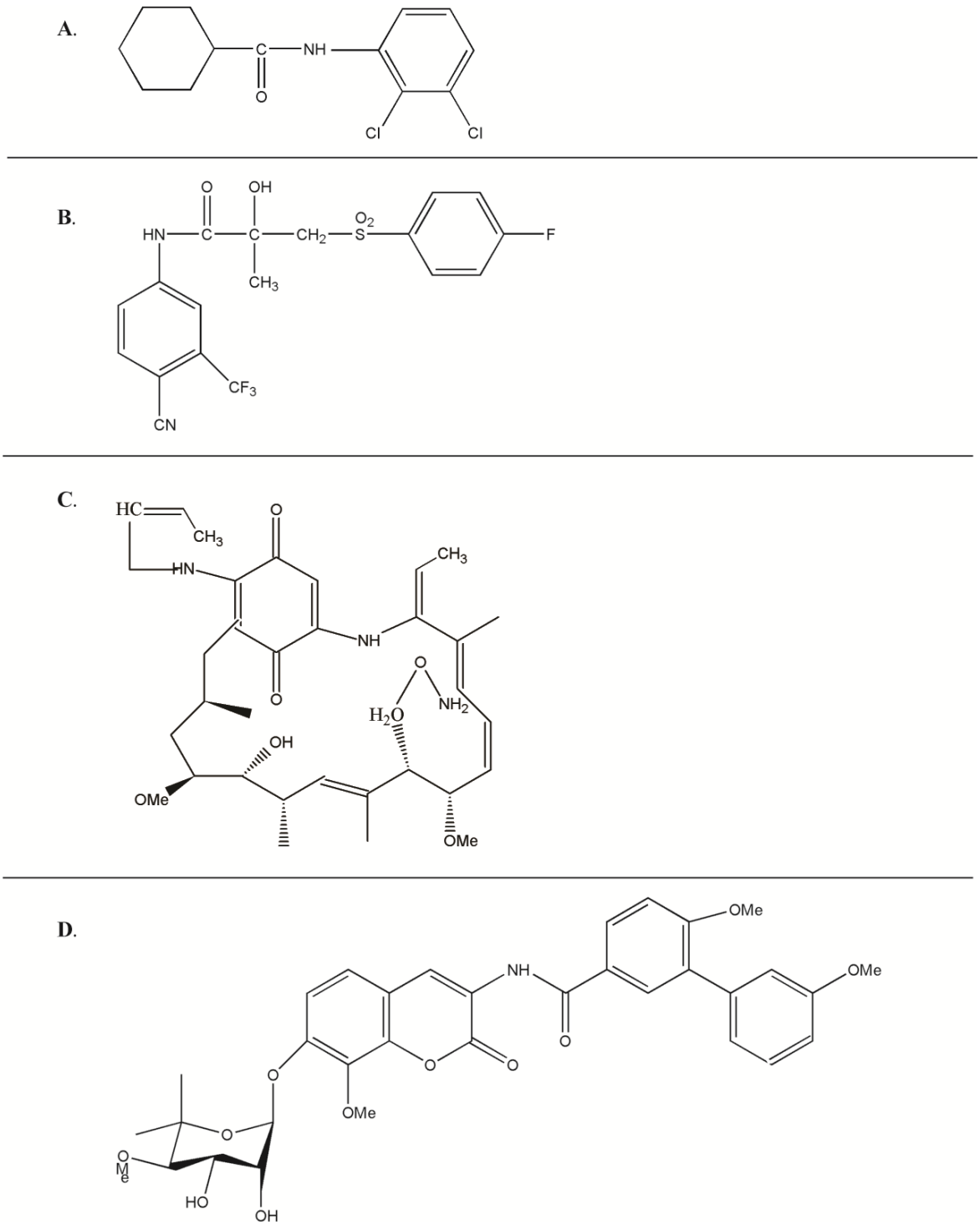



Figure 3.1: Chemical Structures of Compounds Utilized

Chemical structures of the compounds MJC13 (A), Bicalutamide (B), 17AAG (C), and KU174 (D) are represented above.

from contacting Hsp90. Meanwhile MJC13 would prevent the interaction of the BF3 surface with FKBP52/ β-catenin. **Figure 3.2** documents the proposed targets of not only these drugs but also drugs to be considered in the future and illustrates the rationale behind the combination approach utilized in this aim.

MJC13's $IC_{50}$ is 0.45uM in 52KO MEF cells co-transfected with AR, FKBP52, and reporter plasmids. In preliminary studies, we co-transfected the above plasmids alongside β-catenin into 52KO MEFs and generated a dose-response curve to see if MJC13's $IC_{50}$ changed in response to the additional β-catenin, but it did not greatly affect the value. *Our initial hypothesis was that MJC13 would block the FKBP52 component of FKBP52/β-catenin synergism more potently if administered in conjunction with Hsp90 Inhibitors or the anti-androgenic drug Bicalutamide.* To investigate this hypothesis, we performed co-transfections of AR, FKBP52, and reporter plasmids into 52KO MEF cells, and then we administered the drug treatment for an hour soak prior to DHT induction or vector administration as follows:

A. A range of MJC13 doses administered with either the $IC_{50}$ dose of 17AAG, KU174, or Bicalutamide

B. A range of doses of 17AAG, KU174, or Bicalutamide administered with the $IC_{50}$ dose of MJC13

Reporter assays would follow to assess dose-response relationships and to determine $IC_{50}$ values.

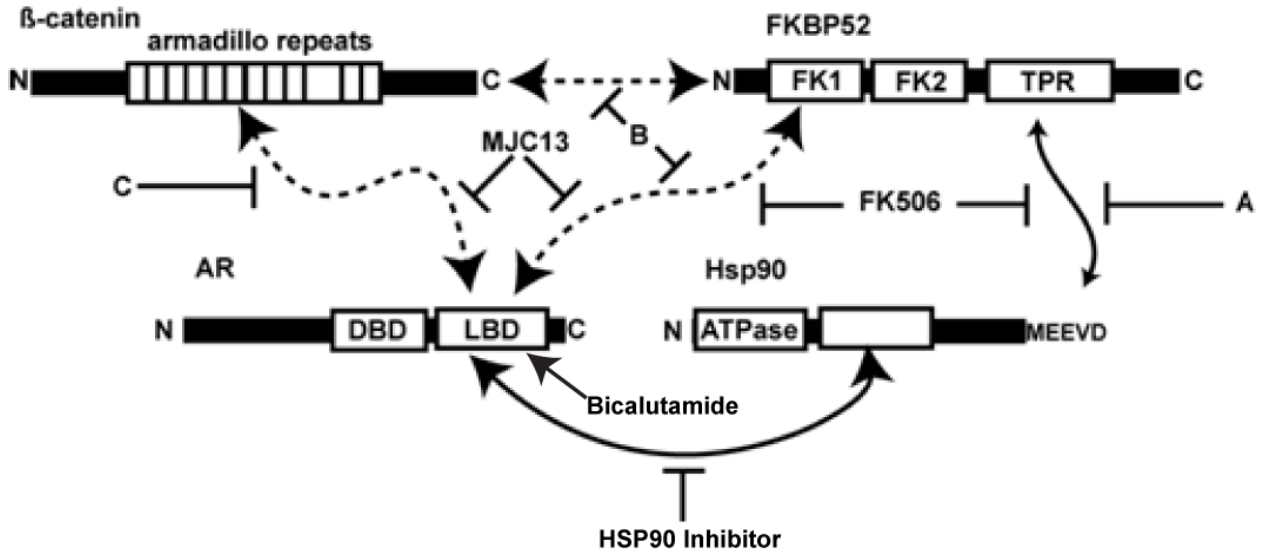



Figure 3.2: Proposed Targets of Drug Combinations

To block FKBP52/β-catenin synergism, three approaches could be taken to block each of the three components. A. The FKBP52/Hsp90 interaction could be targeted through a drug such as FK506. B. An AR BF3 surface inhibitor such as MJC13 could prevent interactions with proteins such as FKBP52 which bind the BF3 surface. C. The β-catenin/AR interaction could be blocked. Additionally, the AR LBD could be inhibited through Bicalutamide to block the ligand from binding receptor or through an Hsp90 Inhibitor such as 17AAG or KU174 which would prevent the receptor from contacting Hsp90.

## 3.2 MATERIALS AND METHODS

For assays, immortalized 52 KO MEF cells were cultured at 5% CO2 in HyClone MEM/EBSS with 2 mM L-glutamine (Thermal Scientific, Logan, UT) supplemented with 10% Charcoal/Dextran Treated FBS (HyClone, Logan, UT) twenty-four hours prior to transfection. Cells were plated at a concentration of 2 $x10^6$ cells/well in 6-well plates at approximately 80% confluence. They were transfected in duplicates using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfections were performed for 5 hours using 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 the 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 either the pCI-Neo plasmid expressing FKBP52, or with 800 ng of pCI-Neo plasmid as a normalizing vector. In the initial study, 800 ng of pCMV-Sport6 plasmid (Open Biosystems, USA) expressing β-catenin was also transfected.

Twenty-four to thirty hours after transfection, medium was replaced and inhibitor was added for a 45 minute to 1 hour soak followed by treatment with DHT (concentrations used corresponded with the $EC_{50}$ of DHT) for 16 hours. Inhibitors were stored in dimethyl sulfoxide (DMSO) and were added individually at a volume equivalent to 0.5% of the media volume in each experiment. For monotreatment studies, a range of doses were given of single inhibitor treatments with the following inhibitors: MJC13 (Sigma Aldrich, St. Louis, MO), Bicalutamide (Sigma Aldrich, St. Louis, MO), 17-AAG (A.G. Scientific, San Diego, CA), or KU174 which was kindly donated by B.S. Blagg, University of Kansas (135). In the first set of combinatorial

inhibitor studies, the $IC_{50}$ dose of the drugs Bicalutamide, 17-AAG, or KU174 was added to each well at a volume equivalent to 0.5% of the media volume simultaneously with a range of doses of MJC13, also at a volume equivalent to 0.5% of the media volume. In the second set of combinatorial inhibitor studies, the $IC_{50}$ dose of the drug MJC13 was added to each well at a volume equivalent to 0.5% of the media volume simultaneously with a range of doses of Bicalutamide, 17-AAG, or KU174, also at a volume equivalent to 0.5% of the media volume.

After approximately sixteen hours of incubation with hormone, cells in each well were lysed using 100μL M-PER (Pierce, Rockford, IL) supplemented with Complete EDTA-free Mini Protease Inhibitor (Roche, Manheim, Germany) and spun using a 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 (incubation time was approximately 5 minutes for the luciferase assay and 2 hours for the β-galactosidase assay), followed by quantification of luminescence by a microplate luminometer (Luminoskan Ascent, Thermo Labsystems). Luminescence was measured in RLU. The transfection-efficiency normalized levels of reporter expression (luciferase RLU/ β-galactosidase RLU) were reported in graphical form using Graphpad Prism software. Data was represented as the mean (+/- standard deviation) of two separate samples, and figures are composite graphs representing data from at least three independent experiments.

## 3.3 RESULTS

### 3.3.1 Drug Profiles for MJC13, 17AAG, KU174, and Bicalutamide in 52KO MEF Cells

The AR BF3 surface inhibitor MJC13 effectively blocks FKBP52/β-catenin synergism in 52 KO MEF cells (**Figure 3.3**). To generate this graph, 52 KO MEF cells were co-transfected with wild type AR, FKBP52, β-catenin, the AR-inducible Probasin luciferase reporter plasmid, and the β-galactosidase reporter plasmid (for normalizing transfection efficiency). Cells were soaked with a range of inhibitor concentrations or DMSO vehicle control for forty-five minutes, followed by induction with the half maximal effective concentration ($EC_{50}$) of DHT for this cell line. Following 16 hour incubation, cells were lysed, AR transcriptional activity was tested through a luciferase assay, and transfection efficiency was normalized through a β-galactosidase assay. This dose-response study was performed in triplicate, and a representative curve was selected as shown in the figure.

To increase the potency of MJC13, drugs 17AAG, KU174, and Bicalutamide were also examined using a combinatorial approach with MJC13. β-catenin addition to the transfection cocktail did not significantly impact the $EC_{50}$ value of MJC13 as reported in literature when only AR and FKBP52 are present (103), and since β-catenin is expressed endogenously in these cells, our studies of drug compounds were conducted solely in the presence of AR and FKBP52.

The Hsp90 inhibitors 17AAG and KU174 were selected primarily for the targeting of Hsp90's other (non-steroid hormone receptor) clients, which might participate in a FKBP52/β-

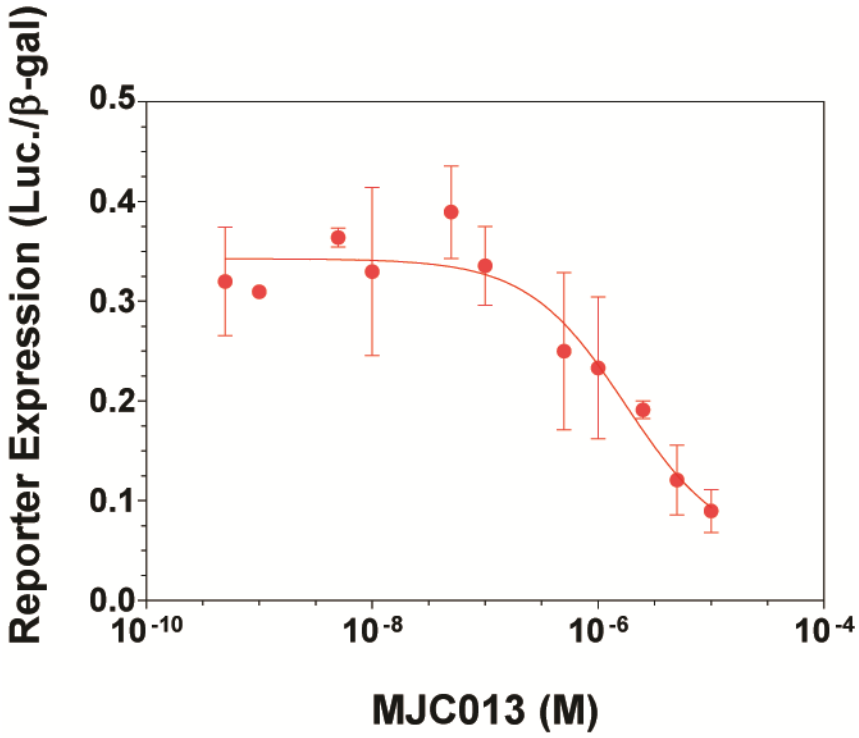



Figure 3.3: The AR BF3 Surface Inhibitor MJC13 Effectively Blocks FKBP52/ β-catenin Synergism in 52 KO MEF Cells

Wild type AR, the AR-inducible luciferase reporter plasmid driven by the Probasin promoter, the constitutively active β-galactosidase reporter plasmid, β-catenin, and FKBP52 were co-transfected simultaneously in 52KO MEF cells. The following day, cells were treated with a range of doses for forty five minutes with MJC13 prior to addition of the $EC_{50}$ dose of DHT. 16 hours after induction, cells were lysed, and AR transcriptional activity was tested through a luciferase assay, followed by a β-galactosidase assay to control for transfection efficiency. Graphs are plotted as reporter expression (AR-induced luciferase activity/β-galactosidase expression) against concentration of inhibitor (plus SDs [error bars] of duplicates). The data shown are representative graphs indicative of at least three independent experiments.

catenin cascade. Because the binding sites as well as profiles of client degradation due to KU174 and 17AAG are different, we wished to employ both inhibitors in our studies with MJC13. Bicalutamide was chosen due to its anti-androgenic activity (138, 139), its prevention of N-C terminal interactions for AR dimerization, its negative effects on coactivator recruitment and binding (140), as well as its implied cross-talk with other pathways that induce apoptosis (141). Lastly, in SW480 cells, Bicalutamide given at the same time as DHT prevents AR/β-catenin interactions (142), but the mechanism of inhibition is not clear. It was believed that the above drugs would integrate inhibition of other pathways in addition to MJC13.

Prior to administering combinatorial therapies of MJC13 with other drugs, each drug was administered individually to establish a baseline dose-response curve for the drug in 52 KO MEF cells. 52 KO MEF cells were co-transfected with wild type AR, FKBP52, the AR-inducible Probasin luciferase reporter plasmid, and the β-galactosidase reporter plasmid (for normalizing transfection efficiency). Cells were soaked with a range of inhibitor concentrations or DMSO vehicle control for forty-five minutes, followed by induction with the half maximal effective concentration ($EC_{50}$) of DHT for this cell line. Following 16 hour incubation, cells were lysed, AR transcriptional activity was tested through a luciferase assay, and transfection efficiency was normalized through a β-galactosidase assay. **Figure 3.4** shows dose-response curves for the compounds MJC13 (**A**), Bicalutamide (**B**), 17AAG (**C**), and KU174 (**D**). Although each drug's dose-response study was performed in triplicate, a representative curve was selected for each drug, as shown in the figure.

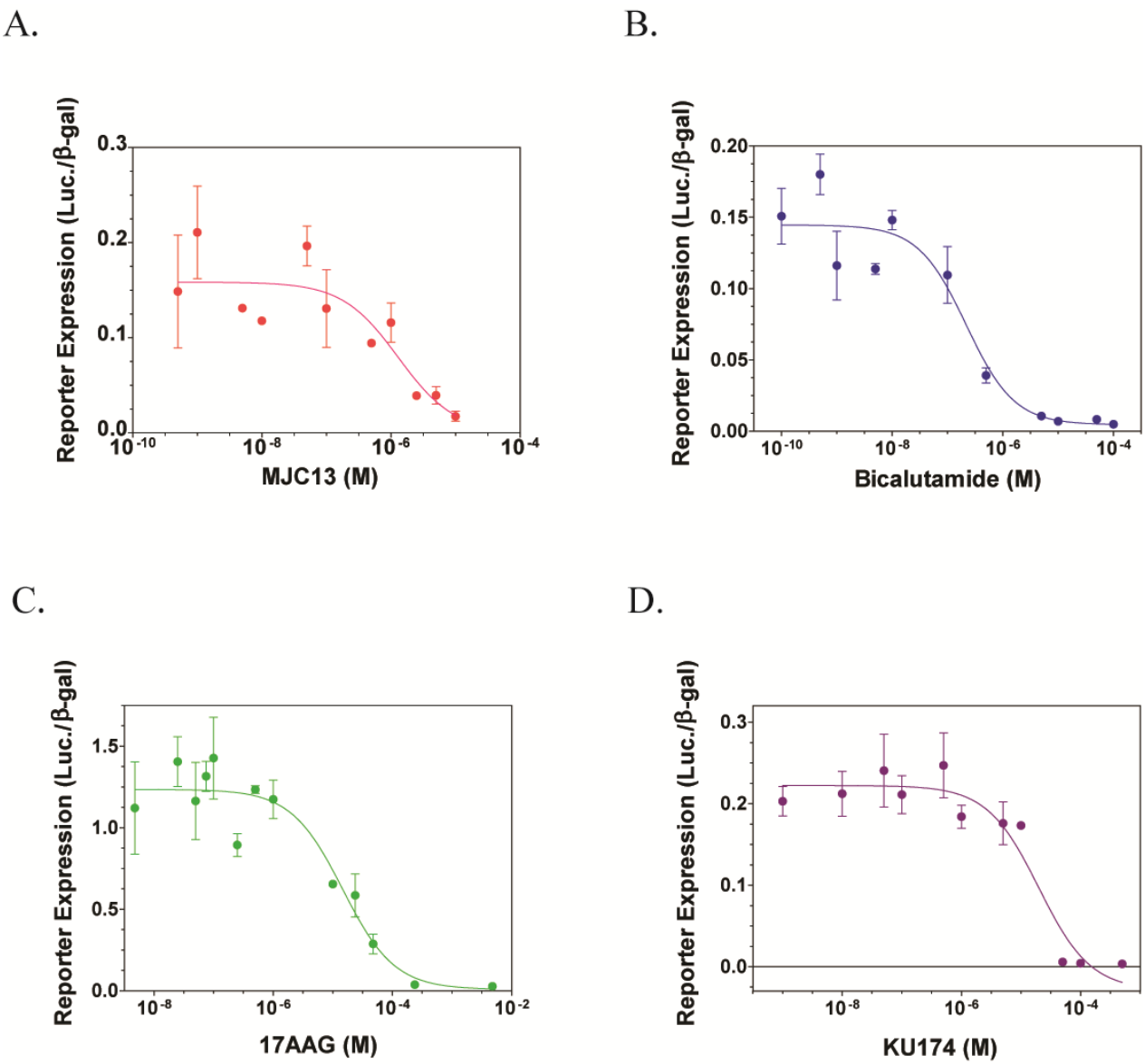



Figure 3.4: Dose-Response Curves of MJC13, Bicalutamide, 17AAG, and KU174 in 52KO MEF Cells Induced with DHT

Figure 3.4: Dose-Response Curves of MJC13, Bicalutamide, 17AAG, and KU174 in 52KO MEF Cells Induced with DHT

Wild type AR, the AR-inducible luciferase reporter plasmid driven by the Probasin promoter, the constitutively active β-galactosidase reporter plasmid, and FKBP52 were co-transfected simultaneously in 52KO MEF cells. The following day, cells were treated with a range of doses for forty-five minutes with the drugs (A) MJC13 (red), (B) Bicalutamide (blue), (C) 17AAG (green), or (D) KU174 (purple) prior to addition of the $EC_{50}$ dose of DHT. 16 hours after induction, cells were lysed, and AR transcriptional activity was tested through a luciferase assay, followed by a β-galactosidase assay to control for transfection efficiency. Graphs are plotted as reporter expression (AR-induced luciferase activity/β-galactosidase expression) against concentration of inhibitor (plus SDs [error bars] of duplicates). The data shown are representative graphs indicative of at least three independent experiments.

### 3.3.2 MJC13 Potency Is Not Improved by the Addition of Bicalutamide, 17AAG, or KU174 $IC_{50}$ Doses in 52KO MEF Cells

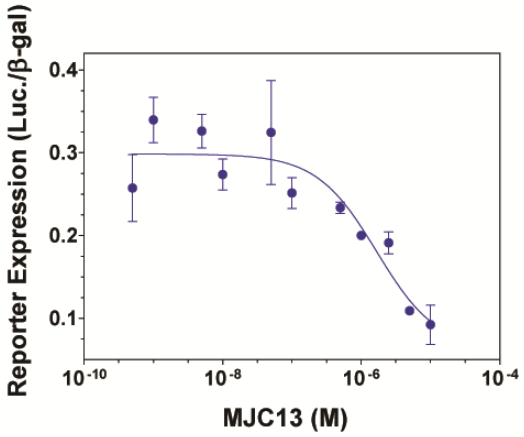
Once $IC_{50}$ values were determined for the compounds Bicalutamide, 17AAG, and KU174, it was then necessary to administer these compounds at their $IC_{50}$ doses simultaneously with different doses of MJC13 to generate a combinatorial dose response curve for MJC13 with each compound. 52 KO MEF cells were co-transfected with wild type AR, FKBP52, the AR-inducible Probasin reporter luciferase plasmid, and the β-galactosidase reporter plasmid (for normalizing transfection efficiency).

Cells were soaked with a range of MJC13 inhibitor concentrations simultaneously with the $IC_{50}$ doses of Bicalutamide, 17AAG, or KU174 (or DMSO vehicle control in place of both drugs) for forty-five minutes, followed by induction with the $EC_{50}$ of DHT for this cell line. Following 16 hour incubation, cells were lysed, AR transcriptional activity was tested through a luciferase assay, and transfection efficiency was normalized through a β-galactosidase assay. **Figure 3.5** shows the resultant dose-response curves for MJC13 with the $IC_{50}$ dose of Bicalutamide (**A**), MJC13 with the $IC_{50}$ dose of 17AAG (**B**), and MJC13 with the $IC_{50}$ dose of KU174 (**C**). Unfortunately, none of these compounds achieved the desired effect. Addition of Bicalutamide or KU174 actually decreased potency by a factor of ten. 17AAG had no effect on MJC13 potency.

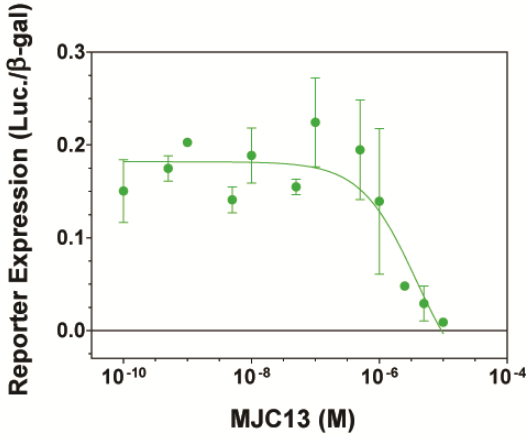
### 3.3.3 Potency of Bicalutamide, 17AAG, and KU174 are not Improved with the Addition of MJC13 $IC_{50}$ in 52KO MEF Cells

While the $IC_{50}$ value of MJC13 did not appear to favorably change in response to Bicalutamide, 17AAG, and KU174, it was still possible that MJC13 $IC_{50}$ dose addition to these compounds could influence their combinatorial dose-response curves.

A.




B.




C.

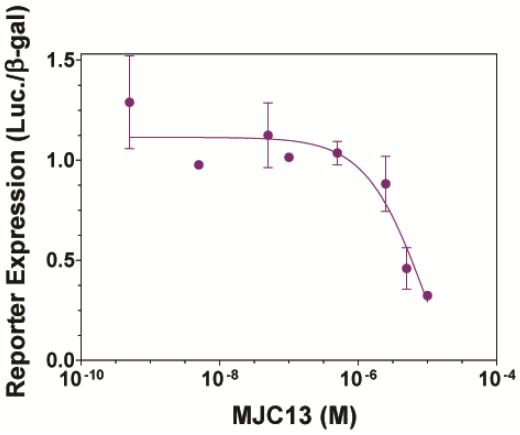



Figure 3.5: Dose-Response Curves for MJC13 in the Presence of $IC_{50}$ Doses of Bicalutamide, 17AAG, and KU174 and the $EC_{50}$ Value of DHT

Figure 3.5. Dose-Response Curves for MJC13 in the Presence of $IC_{50}$ Doses of Bicalutamide, 17AAG, and KU174 and the $EC_{50}$ Value of DHT

Wild type AR, the AR-inducible luciferase reporter plasmid driven by the Probasin promoter, the constitutively active β-galactosidase reporter plasmid, and FKBP52 were co-transfected simultaneously in 52KO MEF cells. The following day, cells were treated with a range of doses for forty five minutes with drug MJC13 alongside the $IC_{50}$ dose of (A) Bicalutamide (blue), (B) 17AAG (green), or (C) KU174 (purple), prior to addition of the $EC_{50}$ dose of DHT. 16 hours after induction, cells were lysed, and AR transcriptional activity was tested through a luciferase assay, followed by a β-galactosidase assay to control for transfection efficiency. Graphs are plotted as reporter expression (AR-induced luciferase activity/β-galactosidase expression) against concentration of inhibitor (plus SDs [error bars] of duplicates). The data shown are representative graphs indicative of at least three independent experiments.

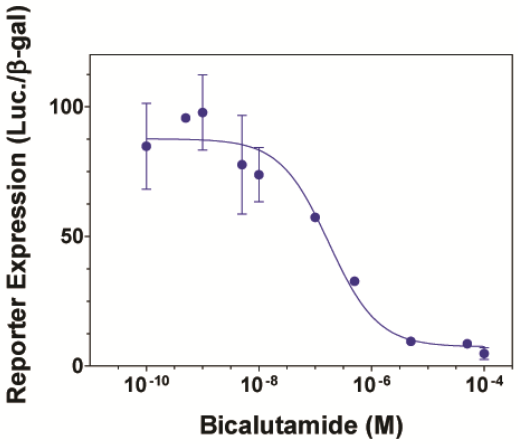
52 KO MEF cells were co-transfected with wild type AR, FKBP52, the AR-inducible Probasin reporter luciferase plasmid, and the β-galactosidase reporter plasmid (for normalizing transfection efficiency). Cells were soaked with the $IC_{50}$ dose of MJC13 inhibitor simultaneously with a range of doses of Bicalutamide, 17AAG, or KU174 (or DMSO vehicle control in place of both drugs) for forty-five minutes, followed by induction with the $EC_{50}$ of DHT for this cell line. Following 16-hour incubation, cells were lysed, AR transcriptional activity was tested through a luciferase assay, and transfection efficiency was normalized through a β-galactosidase assay.

**Figure 3.6** shows the resultant dose response curves for the $IC_{50}$ dose of MJC13 with a range of Bicalutamide doses (A), the $IC_{50}$ dose of MJC13 with a range of 17AAG doses (B), and the $IC_{50}$ dose of MJC13 with a range of doses of KU174 (C). Unfortunately, Bicalutamide, 17AAG, and KU174 did not show significant shifts in potency with the addition of the $IC_{50}$ dose of MJC13.

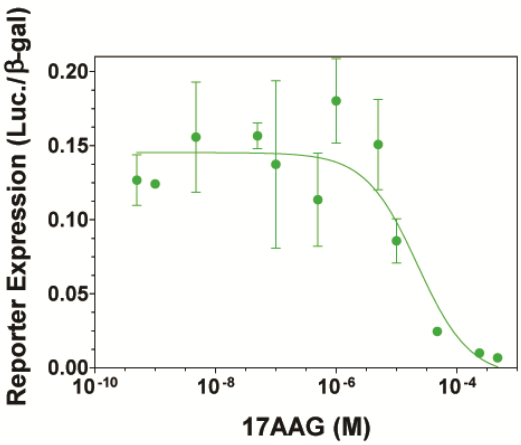
### 3.4 DISCUSSION

The results of all inhibitory studies are visualized in **Table 3.1.** There was clearly redundancy of pathway involvement in many cases because there was no improvement in $IC_{50}$ in the combinatorial studies. MJC13's dose-response curve stayed the same with the addition of 17AAG **(Figure 3.5B)**, but it got worse with the addition of Bicalutamide (**Figure 3.5A**) and KU174 (**Figure 3.5C**). It was also seen as Bicalutamide, 17AAG, and KU174 dose-response curves did not show any potency shifts in response to MJC13 $IC_{50}$ addition **(Figure 3.6).**

A.




B.




C.

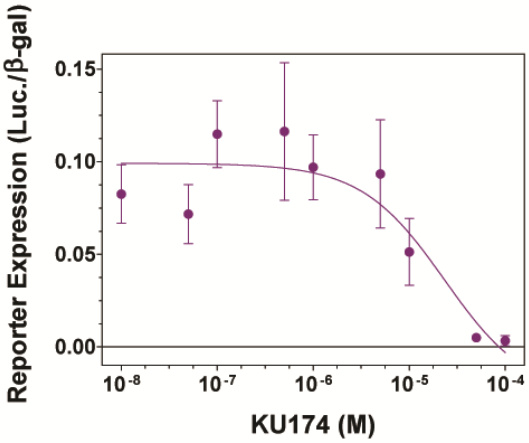



Figure 3.6: Dose-Response Curves for Bicalutamide, 17AAG, and KU174 in the Presence of $IC_{50}$ Doses of MJC13 and the $EC_{50}$ Value of DHT

Figure 3.6: Dose-Response Curves for Bicalutamide, 17AAG, and KU174 in the Presence of $IC_{50}$ Doses of MJC13 and the $EC_{50}$ Value of DHT

Wild type AR, the AR-inducible luciferase reporter plasmid driven by the Probasin promoter, the constitutively active β-galactosidase reporter plasmid, and FKBP52 were co-transfected simultaneously in 52KO MEF cells. The following day, cells were treated with a range of doses for forty-five minutes with a range of doses of (A) Bicalutamide (blue), (B) 17AAG (green), or (C) KU174 (purple) alongside the $IC_{50}$ dose of MJC13, prior to addition of the $EC_{50}$ dose of DHT. 16 hours after induction, cells were lysed, and AR transcriptional activity was tested through a luciferase assay, followed by a β-galactosidase assay to control for transfection efficiency. Graphs are plotted as reporter expression (AR-induced luciferase activity/β-galactosidase expression) against concentration of inhibitor (plus SDs [error bars] of duplicates). The data shown are representative graphs indicative of at least three independent experiments.

Table 3.1: Half Maximal Inhibitory Concentrations of MJC13, Bicalutamide, 17AAG, and KU174 in Isolation and in Combination

| **Drug(s) in Dose Response** | **$IC_{50}$** | **Standard Deviation** |
|---|---|---|
| MJC13 | $1.275x10^{-6}M$ | $2.433x10^{-7}M$ |
| Bicalutamide | $1.839x10^{-7}M$ | $4.865x10^{-8}M$ |
| Bicalutamide $IC_{50}$ + MJC13 | $1.327x10^{-5}M$ | $1.183x10^{-5}M$ |
| Bicalutamide + MJC13 $IC_{50}$ | $5.287x10^{-7}M$ | $6.566x10^{-7}M$ |
| 17AAG | $1.890x10^{-5}M$ | $5.933x10^{-6}M$ |
| 17AAG $IC_{50}$ + MJC13 | $3.266x10^{-6}M$ | $7.057x10^{-7}M$ |
| 17AAG + MJC13 $IC_{50}$ | $1.141x10^{-5}M$ | $1.543x10^{-5}M$ |
| KU174 | $1.559x10^{-5}M$ | $6.102x10^{-6}M$ |
| KU174 $IC_{50}$ + MJC13 | $1.040x10^{-5}M$ | $1.230x10^{-6}M$ |
| KU174 + MJC13 $IC_{50}$ | $1.481x10^{-5}M$ | $1.212x10^{-5}M$ |

This table summarizes the results of all inhibitor trials in Chapter 3 for the drugs Bicalutamide, 17AAG, and KU174, administered alone, dosed as the $IC_{50}$ in combination with a range of doses of MJC13, and administered in a range of doses in combination with MJC13 $IC_{50}$. Average $IC_{50}$ values of all drug trials and the standard deviations of each condition, representing at least three tests per drug performed in duplicates, are shown above.

Addition of the $IC_{50}$ dose of either Bicalutamide or KU174 to MJC13 actually increased the $IC_{50}$ value of the MJC13 curve thereby decreasing its potency to a value tenfold less than MJC13's $IC_{50}$. This was and a hundred-fold less than Bicalutamide's potency and equal to the potency of KU174 (**Figure 3.5A and C**). It is possible that Bicalutamide binding to the LBD of AR affects the orientation of BF3, and that MJC13 binding to BF3 affects the LBD conformation. Therefore, the affinity of the opposing binding surface on AR for the compounds Bicalutamide and MJC13 could be allosterically reduced by the binding these compounds individually to the LBD or BF3 surfaces. As for the KU174 study, one of the known Hsp90 clients is GSK3β, a serine/threonine kinase, which requires Hsp90 chaperone activity to auto-phosphorylate and achieve its mature conformation for activity (143). In targeting these GSK3β intermediates via Hsp90 inhibition, we hypothesize that there could be a decrease in GSK3β activity. GSK3β is responsible for phosphorylation of free β-catenin, which results in β-catenin's degradation and the prevention in its participation in nuclear events (70). In inhibiting Hsp90 chaperoning of GSK3β, even more, free β-catenin could be available to participate in FKBP52/ β-catenin synergy, thereby driving the synergistic reaction on AR expression and counteracting the inhibitory effects of MJC13.

It is curious that 17AAG and KU174, both Hsp90 inhibitors with similar potency in 52 KO MEFs when administered in monotherapy (**Figure 3.4C and D**), did not have the same effects on the MJC13 dose response. 17AAG $IC_{50}$ addition to the MJC13 range of doses had no effect on the MJC13 dose response yet KU174 $IC_{50}$ addition to the MJC13 range of doses decreased potency. Since it has been previously shown that there are different mechanisms of actions for these drugs, it is logical that they could perform differently (135). However, these drugs showed similar effects when administered in a range of doses in conjunction with the $IC_{50}$

dose of MJC13. The addition of MJC13 $IC_{50}$ with doses of 17AAG or KU174 had no significant effects on the dose responses of these compounds (**Figure 3.6 B and C**).

In summary, we believe there are several redundant mechanisms between these drugs. Because 17AAG acts to stop ATP hydrolysis in Hsp90, it would be expected to prevent the release of AR from the complex (108). MJC13 freezes the AR complex in the cytoplasm (103). Since Hsp90 is not believed to participate in nuclear events, the overlapping mechanisms are feasible. The mechanism of action for KU174 is still being determined, but it certainly binds at the C-terminus of Hsp90 to exert effects (135). Bicalutamide, in functioning primarily as an anti-androgen, could have redundant function in that it prevents androgen binding to AR. This also would indicate a cytoplasmic preference for AR, much like MJC13.

Although the graphs are not shown, preliminary studies in which β-catenin was also transfected in conjunction with the Hsp90 inhibitors displayed similar dose response curves and $IC_{50}$ values to the ones seen in this chapter when FKBP52 was transfected without β-catenin. It should be noted that endogenous β-catenin is present in the cells used for the studies shown. Thus, any affects observed upon overexpression of FKBP52 are likely to include coregulation with endogenous β-catenin. We showed in Chapter 2 that FKBP52/β-catenin synergism could occur independently of Hsp90, so it is logical that the Hsp90 drugs might not have much effect in conjunction with MJC13 in blocking synergism.

# Chapter 4: FKBP52/ β-catenin Synergism in Prostate Cancer Cells

## 4.1 RATIONALE

Though the majority of our functional studies of FKBP52/β-catenin synergism of AR had been conducted in 52KO MEF cells, we needed to validate our findings from non-cancerous pilot studies and establish their significance in prostate cancer cells, as well. *Our hypothesis that we wanted to test was that FKBP52/β-catenin synergism of AR would exist in prostate cancer cell lines*. Our first goal was to generate FKBP52 and β-catenin knock down cells in both androgen-dependent and androgen-independent prostate cancer lines. We chose to employ LNCaP, VCaP, LAPC4, and 22RV1 prostate cancer lines to knock down FKBP52 expression. Upon successful knock down, we planned to examine AR response to additional β-catenin transfection when FKPB52 was expressed at normal levels versus at decreased levels using assays comparing cells transfected with β-catenin or vector in control cells expressing FKBP52 versus the FKBP52 knock down cells. This would allow us to determine if synergism occurs in the prostate cancer cells as well as to help us to dissect the roles of FKBP52 and β-catenin as individual components of the synergism.

LNCaP cells are hormone-dependent and were initially isolated from a needle aspiration biopsy of a prostate cancer patient with metastasis to the lymph node. They represent one of the first cellular models of prostate cancer (144). LNCaP cells contain the AR-T877A mutation, which causes promiscuity in binding other hormones (145, 146). They express wild type p53 and also express cytokeratin (CK) 8 and 18. These cells also are ER positive (144) and phosphatase and tensin homolog (PTEN) positive (147).

VCaP, androgen-independent cells, were initially isolated from a patient with vertebral metastasis of hormone refractory prostate cancer and grown in severe combined immunodeficiency (SCID) mice xenografts. They possess wild type AR, a mutant p53 gene

(A248W), and the CKs 8 and 18. They have a broad range of aneuploidy, from hypodiploid to hypertryploid chromosomes, and many have lost their Y chromosome (144). Additionally, AR is overexpressed in these cells; mRNA and protein expression levels of AR are ten times greater in VCaP than LNCaP cells (148).

LAPC-4 is an androgen-dependent cell line derived from lymph node metastasis from a Stage D prostate cancer patient (149). Cells contain wild-type AR (150), a multi-mutant p53 protein (A175H, P72R, R175H), and express CKs 5, 8, and 18. They exhibit triploid karyotypes and lack chromosomes Y and 16 (149). Additionally, LAPC-4 cells are PTEN negative (147). PTEN is a known inhibitor of the Akt pathway (151). Interestingly, β-catenin feeds into the Akt pathway, which is activated by PTEN loss, so this would be an interesting line in which to investigate β-catenin-induced synergism (152).

The 22RV1 cell line, a semi-androgen-dependent line, was originally generated through implantation of an androgen-dependent human prostate cancer tumor in CWR22 xenografts (153, 154), which were serially propagated. The mice were castrated, and 3-10 months later, new tumors arose which were androgen-independent due to mutations of the AR alleles (155). Cells from this xenograft model were harvested and developed into the 22RV1 line for use as a representative *in-vitro* model system for castrate-resistant human prostate cancer (156). This cell line contains a constitutively active form of the androgen receptor, which lacks the ligand binding domain. In the 22RV1 cell line, AR truncation mutants are generated through cleavage by calpain-2. It appears that a mutation to one of the alleles of the gene has caused the insertion of genes near the site of calpain cleavage, enhancing the occurrence of receptor cleavage. Interestingly, some of the human castrate-resistant prostate cancer tissues possess similar truncated mutants, but not all do (157). Additionally, in the full length AR, the H874Y mutation

is present. 22RV1 do not respond as robustly to testosterone as the parent CWR22-dependent cell line. The p53 gene has a Q331R mutation, CK-8 and 18 are expressed, and the line his high expression of Epidermal Growth Factor (EGF) Receptor members and responds both to androgens and EGF. There is cancer-like genomic instability in this line as low passage cells are hyperdiploid and higher passage cells are tetraploid (149).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Generation of *FKBP52* Knockdown Prostate Cancer Cells

22RV1 and VCaP cells were obtained commercially from the American Type Culture Collection (ATCC, Manassas, VA). LNCaP cells were a kind gift from Donald Tindall (Mayo Clinic, Rochester, MN). LAPC-4 cells were obtained from Charles Sawyers (Memorial Sloan-Kettering Cancer Center, New York, NY) and Robert Reiter (Geffen School of Medicine, UCLA, Los Angeles, CA). Cells were maintained in the presence of 10% FBS (Atlas Biological, Fort Collins, CO), and at 5% CO2 at 37 °C. LNCaP and 22RV1 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium with 25mM HEPES with L-Glutamine (Thermal Scientific, Logan, UT). VCaP and LAPC-4 cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) medium with 4 mM L-Glutamine, 4500 mg L-Glucose, and lacking sodium pyruvate (Thermal Scientific, Logan, UT). Cells were supplemented with the following reagents, when not provided in medium: 0.5% HEPES (Thermal Scientific, Logan, UT), 1% Pyruvate (Thermal Scientific, Logan, UT), 1% MEM-non essential amino acids (Thermal Scientific, Logan, UT), 1% Pen-Strep (Thermal Scientific, Logan, UT), and 1% L-Glutamine (Thermal Scientific, Logan, UT).

To generate knockdown cells, all cancer cell lines were maintained in cell culture as described above, and the pSilencer 2.1 U6 Hygromycin (Ambion, Austin, TX) plasmid was sub-cloned with the shRNA cassette against FKBP52 from the old pSilencer vector as well as the scrambled sequence as previously generated by Dr. Cox (11). Cells were infected with lentivirus per instructions for Mission TR Lentiviral System (Sigma, St. Louis, MO). Briefly, cells were plated in 96 well plates and allowed to attach in the 37°C, 5%$CO_2$ incubator. Media was removed and cells were primed with media supplemented with hexadimethrine bromine to increase viral uptake. Viral particles and vector were added, and the mixture was incubated overnight in the absence of Hygromycin. After 24 hours, the media was removed, and clones were selected by repeated cycles of pooling and plating in 150 μg/μL hygromycin every 3 days. Hygromycin concentration for selection was determined by titration prior to the beginning of the experiment.

### 4.2.2 Western Blot of Cellular Lysates

To assess efficiency of knockdown of FKBP52, Western immunoblots were performed using the prepared lysates of control 22RV1 cells and knockdown 22RV1 cells, harvested with MPER as described above. After quantifying protein concentrations of each cell lysate, an equivalent amount of protein was loaded for each sample lane on Criterion gels (BioRad, Hercules, CA), and proteins were separated by electrophoresis. The proteins were then transferred from the gels to Immobilon PVDF membranes (Millipore, Bedford, ME), and the membranes were blotted for protein expression. The following mouse monoclonal antibodies were used as primary antibodies: anti-FKBP52 Hi52d (epitope in FK1 domain) and anti-glyceraldehyde-3-phosphate dehydrogenase (Biodesign International, Saco, ME) as a loading control. The rabbit polyclonal antibody anti-AR (N-20 Santa Cruz, Santa Cruz, CA) was used as

primary antibody to detect AR. Anti-rabbit and anti-mouse secondary antibodies (Southern Biotechnology, Birmingham, AL) were used, and the ImmunStar Alkaline Phosphatase Substrate (BioRad, Hercules, CA) was applied prior to exposing to X-ray films for development and detection of antibodies.

### 4.2.3 Assays in 52 Knockdown 22RV1 Cancer Cells

Twenty-four hours prior to experiments, the 22RV1 control and 22RV1 knockdown cells were plated in RPMI-1640 medium with 25mM HEPES with L-Glutamine (Thermal Scientific, Logan, UT) supplemented with 10% Charcoal/Dextran Treated Fetal Bovine Serum (HyClone, Logan, UT). Cells were plated at a concentration of 2 x $10^6$ cells/well in 6-well plates at approximately 80% confluence.

They were transfected in duplicates 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) and 400 ng of the pT81 (American Type Culture Collection, Manassas, VA) plasmid expressing firefly luciferase reporter driven by the androgen-dependent Probasin promoter. For the β-catenin study in part C, in addition to these plasmids, 800 ng of either the pCI-Neo plasmid (Promega, Madison WI) as a vector control or 800 ng of the pCMV-Sport6 plasmid (Open Biosystems, USA) expressing the S33A β-catenin (mutated as previously described) were also cotransfected.

Twenty-four to thirty hours after transfection, medium was replaced with medium containing DHT or ethanol vehicle control. For part B, a range of doses was used but in part C,

175pM DHT was used to induce cells. After approximately sixteen hours of incubation, cells in each well were lysed using 100μL M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) supplemented with Complete EDTA-free Mini Protease Inhibitor (Roche, Manheim, Germany) and spun using a 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 (incubation time was approximately 5 minutes for the luciferase assay and 2 hours for the β-galactosidase assay), 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 all data was normalized to 100% expression. All data was represented as the mean (+/- standard deviation) of at least two separate samples, and figures are composite graphs representing data from at least three independent experiments.

## 4.3 RESULTS

### FKBP52 Is Required for β-catenin Coactivation of AR in 22RV1 Prostate Cancer Cells

To corroborate our 52 KO MEF studies in prostate cancer cells, we needed to generate a FKBP52-deficient cell line. The 22RV1 prostate cancer cell line contains two forms of the AR gene (**Figure 4.1.A**).

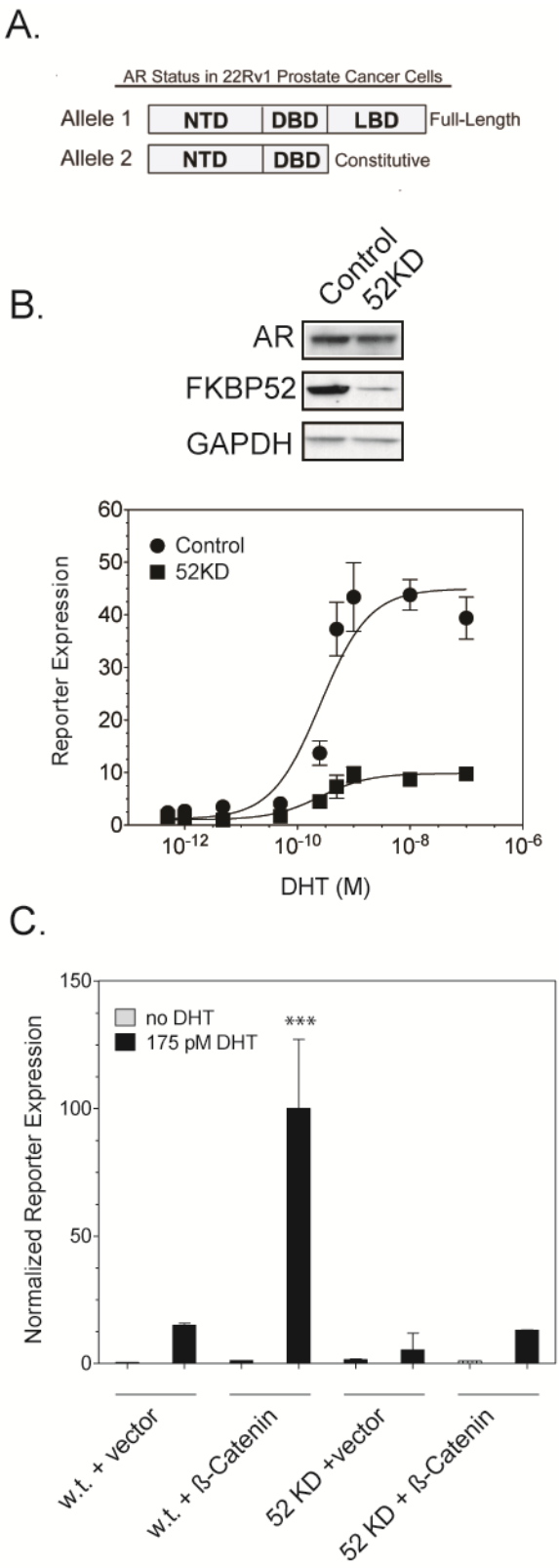



Figure 4.1: FKBP52 is Required for β-catenin Coactivation of AR in Prostate Cancer Cells

Figure 4.1: FKBP52 Is Required for β-catenin Coactivation of AR in Prostate Cancer Cells

A. Knockdown FKBP52 in AR-dependent prostate cancer cells is difficult due to a loss of AR activity and cell proliferation. The constitutively active, truncated AR in 22RV1 supports AR-dependent growth enough to generate cell knockdowns.

B. Scrambled and FKBP52 shRNA constructs were stably transfected into 22RV1 cells using a lentiviral expression system. The selected clones were assessed by Western blot for expression of the indicated proteins. A DHT dose response curve was generated for control and 52KD 22RV1 cells, data points represented as circles and squares, respectively. The data are averages (plus SDs [error bars]) of four independent experiments.

C. The AR-inducible luciferase reporter plasmid and the constitutively active β-galactosidase reporter plasmids were co-transfected simultaneously with either vector or β-Catenin, as indicated, into 22RV1 control or 22RV1 52 KD Cells. Cells were induced with 175 pM DHT (black bars) or ethanol control (gray bars). Following cell lysis, AR transcriptional activity was tested through a luciferase assay, followed by a normalizing β-galactosidase assay. Bars represent luciferase expression/β-galactosidase expression, normalized to the highest signaling group. The data are averages (plus SDs [error bars]) of four independent experiments. Using the Bonferroni statistical analysis follwing a one way Anova test with an alpha value of 0.05, Probasin reporter activity is significantly enhanced in DHT treated 22RV1 control cells transfected with S33A β-Catenin as compared to those transfected with vector, with a p-value < 0.001 (***). There was no statistical significance in the 22RV1 control cells transfected with S33A β-Catenin as compared to those transfected with vector when DHT was not utilized. There was also no statistical significance in 52KD cells transfected with S33A β-Catenin as compared to those transfected with vector, when induced by DHT or treated with vector control.

One allele is full-length but the other allele is a C-terminal truncated form,which is constitutively active (158). In this line, the full-length AR has a mutation affecting the second zinc finger of AR due to an in-frame tandem duplication of exon 3 (144, 149).

To generate the knock down cells, short hairpin RNA (shRNA) that specifically targets FKBP52 mRNA (52KD) or a scrambled shRNA (control) was constructed into a lentiviral vector, and introduced in conjunction with virus to the 22RV1, VCAP, LNCaP, and LAPC-4 cells. Six clones were generated for the 22RV1 cell line only. All other knockdown cells were not viable. Real time PCR (RT-PCR) showed >90% reduction in mRNA for the clone chosen for this study (data not shown). Cell extracts were prepared from each of the cell lines and a Western Blot was performed for AR, FKBP52, or GAPDH as a loading control. In the 22RV1 knock down cell line, FKPB52 expression was reduced to 20% as compared with the scrambled sequence, with no changes in overall AR expression or in expression of the housekeeping gene GAPDH (**Figure 4.1.B**).

Of the prostate cancer lines attempted for knockdown of FKBP52, only the 22RV1 line was able to withstand the effects of *FKBP52* knockdown due to the loss of AR activity and cell proliferation. It is believed that 22RV1's constitutively active AR allows for FKBP52-deficient growth, but the other prostate cancer lines cannot endure this decrease in AR activity. While this serves as validation of FKBP52 as an attractive therapeutic target, it also means that 22RV1 cells are the only prostate cancer cell line in which FKBP52 can be knocked down of the lines we attempted.

Regarding the β-catenin knockdown cells, despite our best efforts, knockdown efficiency was very poor. RT-PCR showed that β-catenin could be knocked down most effectively in 22RV1 where efficiency was 50%. We chose not to pursue further studies with these cells.

Prior to any β-catenin studies in these cell lines, the 22RV1 control and knock down lines were examined for DHT dose responses. To generate these curves, 22RV1 control and 22RV1 52KD cells were cotransfected with the hormone-responsive Probasin luciferase plasmid and the β-galactosidase reporter plasmid for transfection normalization. Transfection of AR is not required in these cells due to its presence endogenously. DHT was administered in a range of doses and allowed to incubate for 16 hours. Following cell lysis, transcriptional activity was assayed through luciferase and β-galactosidase assays. Data points represent expression of the Probasin luciferase reporter normalized to β-galactosidase expression plotted against concentration of DHT. The graph was selected as a representation of three independent experiments, performed in duplicate. In these studies, the $EC_{50}$ of the hormone within both cell lines was approximately 300 pM, and the 20% maximal effective concentration ($EC_{20}$) of the hormone of both cell lines was approximately 175 pM. We chose to employ the $EC_{20}$ value for our transfection studies to characterize synergism.

To determine the importance of FKBP52 in β-catenin regulation of receptor, both control and 52KD 22RV1 lines were used in transfection studies with or without cotransfection of S33A β-catenin. Because FKBP52 expression is greatly reduced in the 52KD line, it serves as a nearly negative background for our studies against the control line, which expresses FKBP52 endogenously. The $EC_{20}$ DHT dose was utilized for minimal hormone induction in these cells. 22RV1 control and 52KD cells were cotransfected with the hormone-responsive Probasin luciferase plasmid, the β-galactosidase reporter plasmid for transfection normalization, and either β-catenin S33A or vector. Reporter gene expression was induced with minimal DHT or vehicle control for 16 hours. Following cell lysis, transcriptional activity was assayed through luciferase and β-galactosidase assays. Bars represent the expression of the Probasin luciferase reporter

normalized to β-galactosidase expression. Reporter expression is expressed as a percentage by dividing normalized luciferase values by the value obtained by the β-catenin transfected control cells when induced by 175 pM DHT, the $EC_{20}$ of DHT.

A comparison of the knockdown groups reveals that in the absence of FKBP52 and the presence of DHT, β-catenin transfection doubles AR signaling. In the absence of hormone, it appears that β-catenin has no enhancement of AR activity, whether FKBP52 is present or not. However, in the wild type group which expresses endogenous levels of FKBP52, DHT addition alone enhances AR activity fifteen-fold over vector. In comparing DHT-induced wild type cells transfected with β-catenin versus those transfected with vector, there is approximately a seven-fold induction. Signaling increases to one hundred-fold over baseline vector (without DHT) when β-catenin is transfected and cells are induced with DHT. This asserts the requirement of FKBP52 for robust β-catenin regulation of AR in 22RV1 prostate cancer cells (**Figure 4.1.C**). Additionally, this result has been repeated by our collaborators at the Methodist Hospital in Houston (Dr. Paul Webb's group) in HeLa cells with and without FKBP52 knock down.

## 4.4 DISCUSSION

The generation of a 52 KD 22RV1 cell line is an accomplishment which will allow our laboratory to continue to investigate FKBP52 interactions more effectively in prostate cancer cells. Because knockdown of FKBP52 was impossible in the other prostate cancer cell lines, but it was effective in 22RV1, it is obvious that this cell line's unique characteristics allowed it to survive, establishing the importance of this essential protein for prostate cancer pathways. Most likely, in the other cell lines, when FKBP52 expression is decreased, AR signaling decreases significantly, leading to the death or decreased proliferation of the cells. In the 22RV1 cells, the constitutively active AR likely allows for survival of the cells in the absence of FKBP52.

While unfortunate that we were unable to generate β-catenin knock down cells, it was not surprising to us that perhaps these prostate cancer lines may not support growth without β-catenin. We were unable to generate knockouts of β-catenin in yeast, so after several months of attempting to knock down this protein in mammalian cells beyond 50% mRNA, we decided to focus our efforts elsewhere.

Our studies of β-catenin transfections in the control versus knock down cells proved the requirement of FKBP52 for β-catenin to regulate AR in the 22RV1 line. When FKBP52 is at 20% of normal expression in this line and cells are induced with DHT, β-catenin transfection barely doubles AR signaling, but in the presence of endogenous levels of FKBP52, β-catenin enhances signaling almost seven-fold. Previous studies in prostate cancer cell lines that deemed β-catenin important for prostate cancer progression did not examine cells that lacked FKBP52.

# Chapter 5: Conclusions

## 5.1 Overall Perspective

In prostate cancer signaling, free β-catenin regulates AR as a result of activation of wnt signaling pathways (82), and some have suggested that activation of the PI3K/Akt pathway may be an upstream regulator of this event. PTEN mutation or loss and WIF-1 down-regulation are two events which increase the phosphorylation-activation of Akt, thereby deactivating GSK3β and allowing cytoplasmic β-catenin to become available to participate in nuclear events with AR (73). Past studies have implicated that β-catenin associates with AR at the AF-2 region of the receptor (81), but our studies have suggested that it specifically associates with the BF-3 surface, as seen in **Figure 2.1**. Additionally, β-catenin is capable of binding AR both when ligand is bound and when it is absent, but DHT addition increases the amount of β-catenin pulled down by AR in co-IPs of LNCaP cells (159)

β-catenin is a well-documented coactivator of AR transcription (81, 82, 85, 159, 160), associating with multiple coactivators and enhancers on promoters. Ligand-bound AR actually competes with TCF4 for regulation by β-catenin (161), thereby diverging away from its normal involvement in the TCF4 pathway (162). β-catenin associates with the TATA box binding protein both in conjunction with the chromatin remodeling complex-associated ATPases Pontin 52 and Reptin 52, as seen by co-immunoprecipitation assays investigating TCF-4's interaction partners (163), and it also associates with Brahma Related Gene 1 (Brg-1), a member of the chromatin remodeling complexes SWItch/Sucrose NonFermentable (SWI/SNF) and chromatin structure remodeling (Rsc) complex as seen in a yeast two hybrid and a co-immunoprecipitation (164). Additionally, β-catenin also associates with the transcriptional co-activator and histone acetylase protein p300 to participate in neoplastic transformation (165). In studying β-catenin's regulation of AR promoters specifically, β-catenin recruits coactivator-associated arginine

methyltransferase (CARM1), a secondary coactivator that is only present on the AR complex when β-catenin is present (166). Additionally, the p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP1) and β-catenin exhibit synergistic activity on AR signaling when co-transfected in CV-1 cells with the MMTV-luc plasmid and AR. While GRIP-1 binds AR directly, it is also capable of binding β-catenin in the absence of AR. Synergistic effects are associated with the GRIP-1 autonomous activation domain 2 (AD2), which is used to bind β-catenin (167).

FKBP52's role as a cochaperone of the Hsp90 complex has been established for decades. FKBP52 potentiates activity of GR (12), PR (13), and AR (11) but not ER or MR (12). FKBP52 increases hormone binding by AR 5-fold over the affinity exhibited when FKBP51 binds AR (45). Few nuclear translocation studies of nuclear receptors have been conducted with AR, specifically; most FKBP52 localization studies have focused on GR. In studies of GR, it appears that FKBP51 is involved in nuclear shuttling of the constitutively active GRβ or hormone responsive GRα when not bound by ligand. Upon ligand binding, a shift in preference of GRα for FKBP52 over FKBP51, a concomitant recruitment of dynein by FKBP52, and the shuttling of the complex to the nucleus via microtubules occurred (168). However, an earlier paper suggested that dynein was not required for FKBP52 to potentiate GR. In this study, a dynein 1-null yeast strain was employed in comparison with a yeast strain expressing dynein. FKBP52 potentiated both strains similarly (12). Another study bolstered the view that FKBP52 regulates nuclear transport. In this study, small interfering RNA (siRNA) of the *fkbp4* gene encoding FKBP52 in SH-SY5Y cells resulted in a noticeable decrease in GR recruitment to the nucleus in response to cortisol administration (169). A recent study suggested that the Hsp90 complex in its entirety could pass through the nucleus. In this study, GR associated directly with Importin B1

(ImpB1), and the Hsp90 complex appeared to favor the association of GR with the importin. Previous studies suggested that upon ligand binding, Hsp90 detached, and the NLS of the receptor was then readily available to cause receptor transport across the nucleus. This lab cross-linked the Hsp90 heterocomplex so that GR could not release from Hsp90 upon ligand binding. When Sf9 cells were permeabilized with digitonin, and dexamethasone hormone was added, the cross-linked complex translocated, showing that transformation (detachment from Hsp90) is not required for GR to travel to the nucleus. Lastly, FKBP52 appears to interact with the glycoprotein Nucleoporin 62 (Nup62) only when in an Hsp90-GR complex. In a reticulocyte lysate immunoprecipitation of Nup62, FKBP52 associated with Nup 62 but FKBP51 did not. When radicicol, the Hsp90 inhibitor, was used, FKBP52 did not associate with Nup62. When radicicol was added to cells and fluorescence microscopy was performed, both FKBP51 and FKBP52 associated perinuclearly, possibly to other nucleoporins. (21). Though the nuclear translocation studies have been conducted in conjunction with GR, FKBP52 could also play a role with AR translocation, possibly through mechanisms involving dynein or Nup62, but since our studies have suggested that FKBP52/β-catenin synergism could occur independently of Hsp90, anything is possible.

Though broad conclusions cannot be produced through the few studies shown here, it is obvious that in the 22RV1 cell model, β-catenin regulates AR very little when FKBP52 is not present (**Figure 4.1**). Therefore, all the previously mentioned studies by which β-catenin acts as a coregulator of AR could indeed have exerted their effects in part due to the synergism exhibited when FKBP52 is present. Additionally, AR associates with proteins such as Hsp90, p23, and FKBP52 (29) in its mature form when it binds hormone. Since our studies have

suggested binding of FKBP52 and β-catenin in the absence of Hsp90 and even AR (**Figure 2.3**), we have found a new mechanism for FKBP52 regulation of receptor involving β-catenin.

### 5.2 PROPOSED MECHANISM

In all the functional studies of β-catenin enhancement of AR activity documented in literature, none have taken place in a FKBP52-deficient cell line. Our studies in 52 KO MEF cells (**Figure 2.2B)** as well as the comparisons of 22RV1 cells against the 52 KD line (**Figure 4.1)** have provided the FKBP52-negative background which was needed to highlight the obligation of FKBP52 for β-catenin to coactivate AR robustly. In the DHT-induced, HeLa Gal4-tk-Luc model which includes endogenous FKBP52, the mere addition of β-catenin causes an 8-fold induction over vector, which is increased to 15-fold with additional FKBP52 expression from transfection (**Figure 2.2A**). When this is compared to a cell line without FKBP52 such as the 52 KO MEF model, however, β-catenin exogenous addition does not significantly regulate the receptor. It is only when FKPB52 is present that β-catenin has an effect, and that effect is synergism (**Figure 2.2B**). This finding is validated through the 22RV1 studies (**Figure 4.1**).

Since it is believed that AR competes with TCF4 for β-catenin binding (161), perhaps the presence of FKBP52 within the AR complex is enough to shift β-catenin binding to favor AR over TCF4. Indeed, ligand-bound AR decreases the transcriptional activity of TCF4 in prostate cancer and in colon cancer (161), and also in neurons (160). Because FKBP52 is overexpressed in prostate cancer patients (40), it seems feasible that overexpression of this protein could be a main reason why β-catenin becomes so active in AR signaling-enhancement in prostate cancer. In a paper assessing anomalies of β-catenin in human prostate cancer tissues, 23% of all hormone-dependent prostate cancer tissues showed free (cytoplasmic or nuclear) state β-catenin.

In almost 39% of all hormone refractory prostate cancer tissues, β-catenin existed in an abnormally free state (170).

When androgen ablation is utilized as a treatment, it has been suggested that β-catenin shifts away from the AR signaling pathway to participate in TCF4 signaling, and this could be the reason for relapse (73). Additionally, acetylation by p300 at K345 on the 6$^{th}$ armadillo repeat of β-catenin causes enhancement of β-catenin/TCF-4 interactions while causing decreased binding affinity for AR. In prostate cancer progression, p300 acetylation could either prevent AR binding directly by β-catenin, as suggested by the Neuveut group (171), or it could alternatively affect FKBP52's binding to β-catenin, thereby favoring the binding of TCF-4 over AR.

Regarding the FKBP52 structural components of synergism, we know that FKBP52's PPIase activity is not required for synergism to occur (**Figure 2.4A**). Also the proline-rich overhanging loop over the PPIase pocket is an important structure for synergism (**Figure 2.4B**). Additionally, FKBP52's K354 residue which was shown to be critical for FKBP52's full regulation of GR and for binding to Hsp90 did not appear to be important for FKBP52/β-catenin synergism (**Figure 2.4C**).

The data presented here suggest that we have discovered a novel pathway by which FKBP52 and β-catenin regulate the androgen receptor independently of the Hsp90 complex. This is supported by the K354A mutant studies (**Figure 2.5C**) and the GST pull-down assays with solely purified β-catenin and FKBP52 (**Figure 2.3**). Our collaborators' findings that the β-catenin-AR interaction is strengthened in the presence of FKBP52 using co-immunoprecipitations suggests that FKBP52 could prime the AR/β-catenin interaction, perhaps

by altering the orientation of the proteins so that there is a stronger interaction between β-catenin and AR.

Although crystal structures have not been isolated for the AR/β-catenin/FKBP52 complex, it is apparent through the studies associated with this project that indeed, this complex is a valid target for prostate cancer studies. β-catenin and FKBP52 might bind together at the BF3 surface of AR to form a complex with high structural integrity (β-catenin and FKBP52 bound as well as individual bonds of β-catenin to AR and FKBP52 to AR).

Initially, my dissertation project solely focused on characterizing the FKBP52 interaction with AR. Through random mutagenesis and site-directed mutagenesis based on nuclear receptor comparisons between receptors regulated by FKBP52 versus those that are not regulated by the immunophilin, we mutated AR to examine its effects on FKBP52 potentiation events. We found a number of AR mutations in what is now called the BF-3 surface of the receptor, which caused receptor dependency upon FKBP52 for signaling (103). However, it is also feasible that FKBP52 may not, in fact, bind AR at BF3 as we initially believed, but rather, it might actually bind β-catenin to help it orient into the correct position to bind the BF3 surface. However, β-catenin does not bind the same subset of nuclear receptors as FKBP52; it only binds AR similarly (82). β-catenin does bind Retinoic Acid Receptor, Vitamin D Receptor, Retinoid X Receptor, PPARγ, and ER. The receptors PR, GR, and Thyroid Receptor interact with Wnt signaling members but not β-catenin directly (172). FKBP52 positively regulates GR (12), PR (13), and AR (11) but not ER or MR (12) as transcription factors. Since β-catenin and FKBP52 share differing interaction partners with the exception of AR, it is still feasible that FKBP52 could still bind BF3 in the case of FKBP52/ β-catenin synergism and is not indirectly binding through β-catenin or Hsp90. Regardless of exact placement of β-catenin and FKBP52 on AR, it

is obvious that they synergize (**Figure 2.2**) and that this synergism can be blocked using the BF3 surface inhibitor MJC13 (**Figure 3.3**).

The MMTV, PSA, and Probasin promoter studies indicated promoter specificity of AR pathway enhancement when FKBP52 and β-catenin are co-transfected (**Figure 2.5**). While these studies are relatively simple, the basic conclusion that can be drawn from these results is profound. Co-regulation of AR activity by FKBP52 and β-catenin is dependent on the DNA sequences on which the receptor acts, suggesting that the regulation is occurring at the level of AR-mediated gene transcription. Not only could this synergism be preferential to particular DNA sequences, but another theory is that differential recruitment of coactivators and corepressors could occur in response to FKBP52/β-catenin/AR interactions. Because the effects are greatest when DHT is present, it suggests that these proteins are synergistic towards AR activation of the gene and not non-specifically or directly influencing transcription of the reporter.

### 5.3 Future Directions

Currently, our collaborators in the Fletterick lab at UCSF, are working to generate a crystal structure of β-catenin and FKBP52 as well as β-catenin, AR, and FKBP52. This information is critical for elucidating the mechanism behind the synergistic effect and to highlighting novel surfaces for the development of therapeutic drug targets for prostate cancer treatments. Effectively designed FKBP52 inhibitors could block FKBP52/AR interactions or FKBP52/ β-catenin interactions to stop synergism.

If a crystal structure is not obtained, we can still employ site-directed mutagenesis of the AR BF-3 surface to target putative binding areas of the receptor based on cross-comparisons of β-catenin and FKBP binding sites on characterized interaction partners. Following mutations,

pull down assays or co-immunoprecipitation reactions can be performed to assess binding to interaction partners. First, we can generate the AR-E893A mutant, which was previously shown to disrupt AR/β-catenin interactions (81). Secondly, based on structures of binding partners of the immunophilin FKBP12 (which has a similar FK1 domain and a strikingly similar open conformation of its proline rich loop to FKBP52), we could mutate exposed Phe or Trp residues on the AR BF3 surface. It has been noted that the immunophilin FKBP12 directly contacts Transforming Growth Factor β (TGF-β) receptor, interacting directly through D37, a residue that is analogous to D68 of FKBP52. When FKBP12 was mutated at either residue 89 or 90 (which corresponds to amino acids 119 and 120 of FKBP52), TGF-β potentiation ability was severely decreased. It also appears that P119 of FKBP12's proline rich loop becomes intercalated between W242 and F243 on TGF-β (45). Since the converse residues of FKBP52 are already established as important functional determinants of AR potentiation and even show importance in synergism (**Figure 2.4B**), we believe that residues of the PPIase domain may serve as interaction surfaces for all FKBPs, and we are interested in finding similar hydrophobic residues on AR, which may bind with FKBP52. We can screen the BF-3 surface for any exposed Phe or Trp residues using 3D protein viewers such as UCSF Chimera (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081)), and we can mutate these residues specifically and perform functional studies in the 52 KO MEF cell line to determine which mutants lost potentiation ability of the receptor, either in conjunction with transfections of AR, β-catenin, and FKBP52; AR and β-catenin, AR and FKBP52; or AR and vector. Binding studies could also be conducted to determine the influence of important residues highlighted in functional studies.

β-catenin interacts with the LBD of the androgen receptor, by virtue of yeast two hybrid screenings (82), mammalian two hybrid screenings (81), and GST pull-down assays (85). X-ray crystallography analyses of β-catenin with LRH-1 suggest possible binding to the BF-3 region of that receptor, and we suspect that it could similarly bind AR. Site-directed mutagenesis of the fifth armadillo repeat, deemed a crucial domain for interaction between β-catenin and LRH-1 (173), was performed as a part of this project (results not shown). These mutations did not appear to abrogate FKBP52/β-catenin synergism. However, mutations to other armadillo repeat domains or other residues besides those in the fifth armadillo repeat could also be examined by both site-directed mutagenesis and random mutagenesis since the other armadillo repeats have also been implicated in binding to proteins such as the transcription factor TCF-4 (81). Random mutants could be screened through yeast reporter assay systems followed by studies of interesting mutants in 52 KO MEF cell line to determine which mutants lose potentiation ability of the receptor, either in conjunction with co-transfections with AR and either FKBP52 or vector. Binding studies could also be conducted to determine the influence of important residues highlighted in functional studies.

Another collaborator, Paul Webb at Methodist University, is performing analyses to assist in our understanding of FKBP52/β-catenin interactions at the DNA level. He is transfecting FKBP52 and or β-catenin into 22RV1 control versus 52 KD 22RV1 cells, cross-linking the coactivator surface of AR to any bound coactivators, and performing ChIP analysis to assess differential recruitment of coactivators and/or alterations to binding sites on DNA in response to FKBP52/β-catenin transfections. Additionally, Dr. Webb is examining genome-wide expression changes in the presence or absence of MJC13.

Jane Trepel, a collaborator with the NIH, is able to monitor RNA and protein expression in prostate tissues from prostate cancer patients. She will help us to conduct RT-PCR analyses of endogenous gene transcription of AR target genes such as PSA in response to transfections with FKBP52 and β-catenin or treatments with MJC13. RNA analyses can be supplemented with protein expression studies and immunohistochemistry analyses to examine translational changes in these tissues.

Since we were not able to generate β-catenin knockdown cells, an alternative means to provide a β-catenin negative background would be to employ inhibitors of β-catenin. Many inhibitors are commercially available, from the inhibitors of Akt/IP3 such as wortmanin or LY294002 to Wnt inhibitors such as administering a secretory LBD of Frizzled receptor, which impact the β-catenin signaling cascade. Or novel inhibitors to β-catenin specifically which only block its interaction with FKBP52 could be developed.

Immunofluorescence microscopy experiments to show the localization of the proteins β-catenin, AR, and FKBP52 within the cell would provide concrete insight into the site of regulation of the receptor. Attempts to perform nuclear fractionation studies of cells under different transfection conditions have proven inconclusive (results not shown). It appears that overall β-catenin expression in the cell is maintained at a constant level, so we theorized that localization changes occurred in response to transfections of the protein. However, nuclear fractionation is not the ideal method to study β-catenin. We are hopeful that confocal microscopy could prove more consistent and definitive.

Recently, AR has been discovered in about 70% of all breast cancer tissues. AR appears to play a critical role in triple negative breast cancers. 25% of all breast cancer patients lack PR, ER, and HER2 receptor but have AR, and it appears that AR is a major activator in these cells

(174). It would also be interesting to conduct transfections followed by assays in these cells to determine if β-catenin, AR, and FKBP52 play a role in this subset of the breast cancer population.

Lastly, as new drugs are developed to target this synergism, it would be important to begin trials of these compounds and to examine FKBP52/β-catenin synergism of AR in prostate cancer mouse xenograft models.

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# Curriculum Vita

Cheryl Storer earned her Bachelor of Science degree in biochemistry from Campbell University in North Carolina in 2004. She attended The University of Alabama at Birmingham to study optometry from 2004 to 2005. 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. Storer has been the recipient of numerous honors and awards at UTEP. She received the HHMI Undergraduate Student Trainer Fellowship and the NSF-GK-12 Fellowship. Additionally, she received a UTEP Graduate School Research Award and the Sun Conference Graduate Student Teaching Portfolio Award in 2010. She has been nominated for the UTEP Outstanding Teaching by a Graduate Assistant Teaching Award by her department in 2013. While pursuing her degree, Dr. Storer worked as a teaching assistant of Immunology, Prokaryotic Molecular Genomics, Molecular Cell Biology, and Organismal Biology, and she worked as a research assistant under Dr. Marc Cox.

Dr. Storer has presented her research at numerous conferences, including poster and oral presentations at the Midwest Stress Response and Molecular Chaperone Meeting in Evanston, IL, a poster presentation at the AACR San Antonio Breast Cancer Symposium, an oral presentation at the UTEP Graduate Research Exposition, an oral presentation at the El Paso Kaleidoscope of Teaching Math and Science, a poster presentation at the NSF GK-12 National Conference, and poster presentations at UTEP. She has been supported in her travel by the UTEP Graduate School, the UTEP College of Science, the UTEP Biology Department, the UTEP NSF GK-12 Program, and the UTEP SGA.

Dr. Storer's publications and co-authorships have been featured in *Trends in Endocrinology and Metabolism, Proceedings of the National Academy of Sciences, and Current Opinions in Pharmacology*. Her final publication is in preparation and should be released in fall 2013.

Her dissertation project entitled "A Novel FKBP52/β-catenin Complex Specifically Regulates Androgen Receptor Activity" was supervised by Dr. Marc B. Cox. Upon graduation, Dr. Storer plans to teach undergraduate biology courses and work with undergraduates on small research projects.

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