Vitamin D Deficiency, MEGALIN and High Prostatic Androgens in African American Men

BY

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THESIS

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DEDICATION

I will first and foremost dedicate this my mother, Gladys Garcia, who's unconditional and relentless love transformed my misled and troubled past into a well-guided and promising future. My mother has and always will remain a pillar of strength and the foundation to my success, and now I will be the same for her. Frankly, I would not have made it this far without you, Mom. As I complete my thesis work, I pray that your steadfast and successful efforts to raise a man of God are revealed to you, and that I am blessed to have a mother like you.

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both have consistently supported me in numerous ways throughout my entire life and as a result, I will do the same for my niece and nephew in my father's absence.

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CONTRIBUTION OF AUTHORS

Figures 4: This work was previously published in JCI Insight (Richards et al., 2017)

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Figure 13-14: Androgen metabolites in these figures were measured by Yue-Ting Wang in the UIC Mass Spectrometry, Metabolomics & Proteomics Facility.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS AND NOMENCLATURE

1,25D	1,25-Dihydroxyvitamin D
25D	25-Hydroxyvitamin D
1,24,25D	1,24,25-Dihydroxycholecalciferol
24,25D	24,25-Dihydroxycholecalciferol
3D	Three-Dimensional
5AR	5α-Reductase
AA	African American
ABP	Androgen Binding Protein
ACTH	Adrenocorticotropic Hormone
ADT	Androgen Deprivation Therapy
AR	Androgen Receptor
ARES	Androgen Response Elements
D2	Cholecalciferol
D3	Ergocalciferol
DBP-488	Alexa - 488 Labeled DBP
DBP	Vitamin D Binding Protein
7DHC	7-Dehydrocholesterol Reductase
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
EA	European American
EnzR	Enzalutamide Resistant
FBS	Fetal Bovine Serum
FHH	Free Hormone Hypothesis
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
LH	Luteinizing Hormone
LRP2	Low Density Lipoprotein-Related Protein 2
PCa	Prostate Cancer
PrE	Primary Epithelial Cells
PSA	Prostate Specific Antigen
RAP	Receptor Associated Protein
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
SES	Socioeconomic Status
SHBG	Sex-Hormone Binding Globulin
SHBG-555	Alexa-555 Labeled SHBG
SRD5A2	Steroid 5 Alpha-Reductase 2
T	Testosterone
TMA	Tissue Microarray
uHPLC-MS/MS	Ultra-high Performance Liquid Chromatography tandem Mass Spectrometry
UIC	University of Illinois at Chicago
UVB	Ultraviolet Radiation-B
VitD	Vitamin D
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
%WAA	Percent West African Ancestry

SUMMARY

Prostate cancer is the most frequent (excluding skin) malignancy diagnosed in males (in the US) and it is estimated that 1 in 7 men will be diagnosed within their life. African American men are inordinately at risk for developing this disease and are twice as likely to die from it compared to European American men. African American men are also susceptible to vitamin D deficiency as melanin inhibits cutaneous synthesis of vitamin D. Vitamin D is a hormone suspected to be chemopreventive in the prostate and its deficiency has been theorized to promote prostate cancer disparities in African American men. Androgens are another class of steroid hormones with carcinogenic properties that promote prostate cancer progression. This thesis challenges the ideas and explores the outcomes of an extracellular receptor, MEGALIN, that can internalize and be negatively regulated transcriptionally by vitamin D and androgens within the prostate.

CHAPTER I: INTRODUCTION

A. Ancestry related disparities in prostate cancer

Prostate cancer (PCa) is currently the second most diagnosed cancer in men in the United States. PCa incidence and mortality rates vary by race, with African Americans (AA) having about 60% increased PCa risk and double the lethality compared to their European American (EA) counterparts (Siegel et al., 2018) . AAs present at an earlier age (Cotter et al., 2002) with more aggressive disease (Hoffman et al., 2001), have higher PSA (Moul et al., 1995), larger tumor volume (Sanchez-Ortiz et al., 2006) and respond less favorably to standard treatments compared to EAs (Smith et al., 2017). The reasons for these disparities are multifactorial, likely involving biological factors as well as differences in lifestyle, screening, treatment, and quality of care in some cases. However, when controlling for socioeconomic factors, the disparity persists with AA men presenting with PCa at a younger age (Nettey et al., 2018; Riviere et al., 2020) and with disease that is more aggressive (Abern et al., 2013)

Income and access to healthcare are well documented socioeconomic factors that impact PCa stage at diagnosis and PCa survival in AA men. (Coughlin, 2020). In a cohort of 61,000 PCa cases, individuals categorized within the lowest quartile of socioeconomic status (SES) displayed a 31% increase in PCa mortality, demonstrating that AA men were significantly more likely to die from PCa compared to Caucasian men (Du et al., 2006). In another cohort of 100,000 men, Cheng et al found that AAs had a two to five-fold increase in PCa mortality compared to non-Hispanic white men across all levels of SES (Cheng et al., 2009). Examining the relationship between SES and PCa stage at diagnosis, Schwartz et al showed those with the highest SES scores had higher chances of presenting with localized disease compared to those who scored the lowest, confirming that SES is contributing to the disparity in stage between AA and EA men (Schwartz et al., 2003). In a pivotal effort to control for SES in a racially diverse cohort of Veterans with equal access to healthcare, a recent retrospective observational analysis demonstrated that AA men did not present

with more aggressive disease or have higher rates of PCa mortality, inferring that when healthcare is provided equally, the disparity in PCa mortality is eliminated. The authors noted that although the results do not support the notion that PCa is inherently more aggressive in AAs, it is likely there remain biological determinants that affect the age of onset and frequency of PCa in AA men. (Riviere et al., 2020).

From 2012-2016, AA men were 1.7 times more likely to develop PCa compared to EA men (Siegel et al., 2018). SES tends to associate positively with PCa incidence (Cheng et al., 2009; Tomic et al., 2018), thus the etiology of this disparity remains largely misunderstood. Although familial history and age remain the greatest risk factors for developing PCa in all men, ancestry-specific molecular and genetic alterations in prostate biology may contribute to this racial disparity. Single nucleotide polymorphisms at the 8q24 locus have been identified to show increased susceptibility to PCa in men of African ancestry compared to European ancestry (Dupont et al., 2020; Freedman et al., 2006; Yeager et al., 2009).

Additionally, the androgen axis, which is known to be involved in PCa initiation and development, and favors increased signaling in AAs compared to EAs (Karakas et al., 2017). AA's have fewer CAG repeats in the androgen receptor (AR), (Bennett et al., 2002; Platz, 2000) a trait that results in more functional AR. Mutations in the SRD5A2 gene, which encodes for the 5α-reductase that converts testosterone (T) to the more potent AR agonist dihydrotestosterone (DHT) **(Figure 1)**, are more frequent among AAs and may confer elevated enzyme activity (Reichardt et al., 1995). AAs also harbor more polymorphisms in the androgen-producing enzyme CYP17A1 (Kittles et al., 2001) and androgen-inactivating enzyme CYP3A4 (Paris et al., 1999; Powell et al., 2004), that have been found to associate with PCa.

B. Vitamin D deficiency, Prostate Cancer, and African Ancestry

In addition to the disparity in PCa, AA men are also at increased risk for vitamin D (vitD) deficiency, which is epidemiologically associated with cancers of the breast, colon, and prostate, (Murphy et al., 2012; Steck et al., 2015). Multiple epidemiological studies in racially diverse cohorts have shown that serum 25-hydroxyvitamin D (25D) levels inversely associate with lethal PCa in AA men (Murphy et al., 2014; Nelson et al., 2017; Nyame et al., 2016; Steck et al., 2015). In the absence of supplementation, vitD status is a result of sun exposure and skin pigmentation, as melanin reduces cutaneous vitD synthesis in response to UVB exposure (Matsuoka et al., 2012). Melanin positively correlates with African ancestry, and therefore increases the risk of vitD deficiency in AAs (Batai et al., 2021; Forrest and Stuhldreher, 2011; Signorello et al., 2010). A study by Murphy et al conducted among AA men living in Chicago IL revealed that AAs are both seasonally deficient throughout the year and have 60% less daily vitD intake (dietary and supplements) compared to EA men. The study concluded that 90% of AAs were vitD insufficient (<30ng/mL) and 60% were deficient (<20 ng/mL) in 25D (Murphy et al., 2012).

Epidemiological evidence of vitD conferring protection against PCa have largely been mixed with results showing positive (Ahn et al., 2008; Xu et al., 2014), negative (Ahonen et al., 2000; Corder et al., 1993), and no association between vitD status and risk (Braun et al., 1995; Gann et al., 1996b; Manson et al., 2019; Travis et al., 2009) Moreover, there is evidence suggesting a U-shaped curve where both low and high levels of 25D associated with increased risk (Kristal et al., 2014; Tuohimaa et al., 2004). Interventional studies with vitD metabolites have reported decreased prostate specific antigen (PSA) levels (Woo et al., 2005), a reduction in the proliferation marker Ki67 (Christakos et al., 2013; Giangreco et al., 2013; Wagner et al., 2013), and decreased number of positive biopsies (Hollis et al., 2013), although these studies did not include a diverse population of AAs. Overall, analyses restricted to vitD status and PCa risk in AA men have remained inconsistent.

C. Vitamin D Metabolism and Chemopreventive Effects

VitD is a steroid hormone essential for normal human physiology that exists in two isoforms: cholecalciferol (D_3) and ergocalciferol (D_2) (Lips, 2006). D_3 is primarily obtained through cutaneous UVB exposure and dietary animal products whereas D_2 is acquired from dietary plant sources. Both forms are biologically relevant, yet the D_3 isoform has an extended half-life, is more efficient in raising and preserving circulating 25D levels and comprises the majority (>90%) of total vitD in most populations (Heaney et al., 2011; Lehmann et al., 2013).

Canonically, vitD has been studied in the context of calcium homeostasis and bone health for which the mechanism is well established (Christakos et al., 2016; DeLuca, 1988). However, vitD exerts diverse pharmacological activity, targeting nearly all tissues and its roles in cellular metabolism, innate and adaptive immunity, and phosphate homeostasis, have also been reviewed comprehensively (Christakos et al., 2016; Christakos et al., 2013; Feldman et al., 2014; Hewison, 2012). VitD has also been shown to exhibit anti-transformative properties in vitro that decrease inflammation and proliferation, inhibit angiogenesis, reduce invasion and metastasis, promote differentiation, and induce apoptosis in a variety of cell types (Bao et al., 2006; Barreto et al., 2000; Feldman et al., 2014; Nonn et al., 2006; Pendas-Franco et al., 2007; Simboli-Campbell et al., 1997; Sung and Feldman, 2000). VitD supplementation in vivo delays the formation of high-grade lesions in genetic mouse models of PCa (Banach-Petrosky et al., 2006) and decreases tumor volume in xenograft models of PCa (Ray et al., 2012; Swami et al., 2012).

VitD synthesis begins with UVB-induced cutaneous isomerization of 7-dehydrocholesterol (7DHC) to D_3. D_3 is then further metabolized through subsequent hydroxylation reactions by cytochrome P450 enzymes yielding both active and inactive metabolites **(Figure 1).** The initial hydroxylation is performed by hepatic CYP2R1 or CYP27A1 where D_3 is hydroxylated at carbon 25, generating the prohormone, 25D. This is the major circulatory form of vitD, and the clinical measure used to assess vitD status. 25D subsequently undergoes 1α-hydroxylation by renal CYP27B1 at carbon 1 forming the active hormone, 1,25-dihydroxyvitamin D (1,25D). Inactivation

of both 25D and 1,25D occurs in the kidney proximal tubule through hydroxylation at carbon 24 by CYP24A1, forming the inactive metabolites 24,25D and 1,24,25D, respectively (Bikle, 2014). Extra-renal tissues express functional hydroxylating enzymes CYP27B1 and CYP24A1, enabling local regulation of vitD metabolites (Giangreco et al., 2015; Hsu et al., 2001; Schwartz et al., 1998).

The pharmacological activity of vitD is achieved through activation of the vitamin D receptor (VDR). The VDR is structurally formed to mediate transcriptional regulation; it contains a DNA binding domain, a ligand binding domain, and is capable of heterodimerization and post-translational modification allowing for recruitment of co-regulators, co-repressors, and fine tuning of its transcriptional activity. VDR is activated by 1,25D, which engages the VDR to form an obligate heterodimer with the unliganded retinoid X receptor (RXR) **(Figure 1**). (Pike and Meyer, 2010.) VDR-RXR recognizes vitamin D response elements (VDREs) throughout the genome triggering activation or repression of target genes through recruitment of coregulator and corepressor machinery and eventually RNA polymerase II (Christakos et al., 2019; Demay et al., 1992).

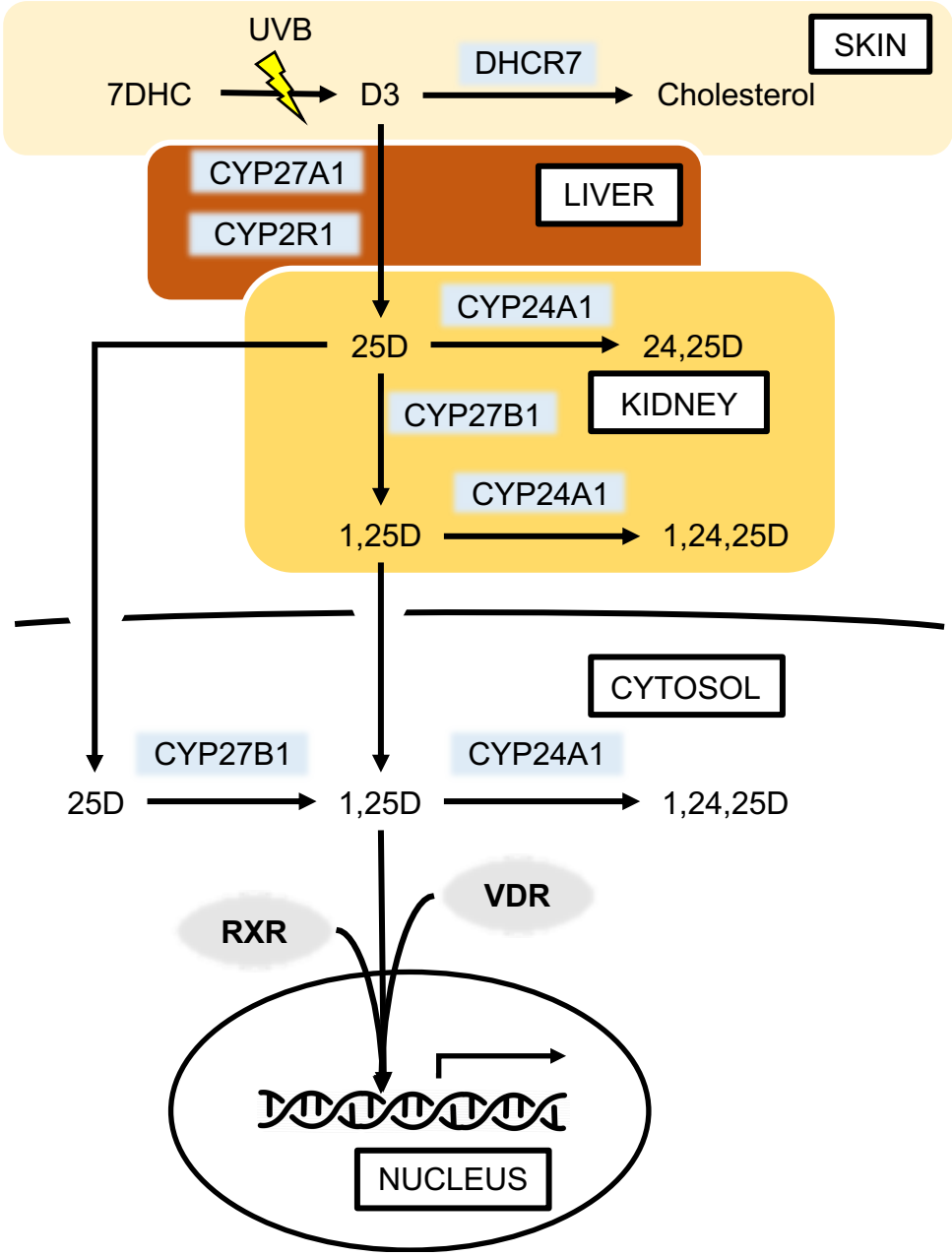

Figure 1 The Vitamin D Axis

The vitamin D axis consists of kidney and liver cytochrome P450 monooxygenases, vitamin D metabolites, extra-renal enzymes, and the vitamin D receptor (VDR).

D. Androgens and Prostate Cancer

Androgens are a class of sex steroid hormones required for growth and reproduction (O'Donnell et al., 2000). The primary androgen in males is testosterone (T), which is the predominant circulating form and precursor to the more potent dihydrotestosterone (DHT) (Nassar and Leslie, 2021). The majority (>95%) of systemic T is produced by interstitial Leydig cells of the testes while the remainder is generated from the adrenal glands, which also secrete dehydroepiandrosterone (DHEA), another abundantly circulating steroid albeit with limited androgenicity. (Brooks, 1975; Mooradian et al., 1987). In circulation, DHT levels are only 10% those of T (Hay and Wass, 2009), and the majority of circulating DHT is generated from the liver with low levels originating from the testes and prostate (Melmed et al., 2015)

Androgen production in humans is governed by the hypothalamic-pituitary-gonadal and adrenal-axes, **(Figure 2)** a diverse organ system of endocrine glands that regulate development and reproduction in humans. In response to low systemic androgen levels in males, gonadotropin-releasing hormone (GnRH) is secreted from GnRH neurons of the hypothalamus to gonadotropic cells in the pituitary gland, which synthesize luteinizing hormone (LH), and follicle stimulating hormone (FSH) (Marieb and Hoehn, 2015). LH binds to the surface of Leydig cells triggering an influx and steroidogenic conversion of cholesterol to T that effluxes into the surrounding tissues and circulation. FSH induces Sertoli cells to secrete androgen-binding protein (ABP) which increases local concentrations of T to enhance its stimulatory effects required for spermatogenesis. The entire system is under tight regulatory control as rising levels of T inhibit the secretion of GnRH and acts directly on the pituitary to disrupt gonadotropin release. When sperm counts are too high, inhibin is released from Sertoli cells to inhibit the secretion of hypothalamic GnRH and pituitary FSH. In addition, hypothalamic release of corticotropin-releasing hormone (CRH) to anterior pituitary corticotropic cells induces production and release of adrenocorticotropic hormone (ACTH) (Ritchie et al., 1996). Pituitary ACTH is then secreted to the adrenal glands

which stimulates production of androgenic biosynthesis of dehydroepiandrosterone (DHEA), another plentiful circuiting androgen precursor, from 17α-hydroxypregnenolone (prohormone) , (Feher et al., 1985; Ganong, 2005)**. (Figure 2).**

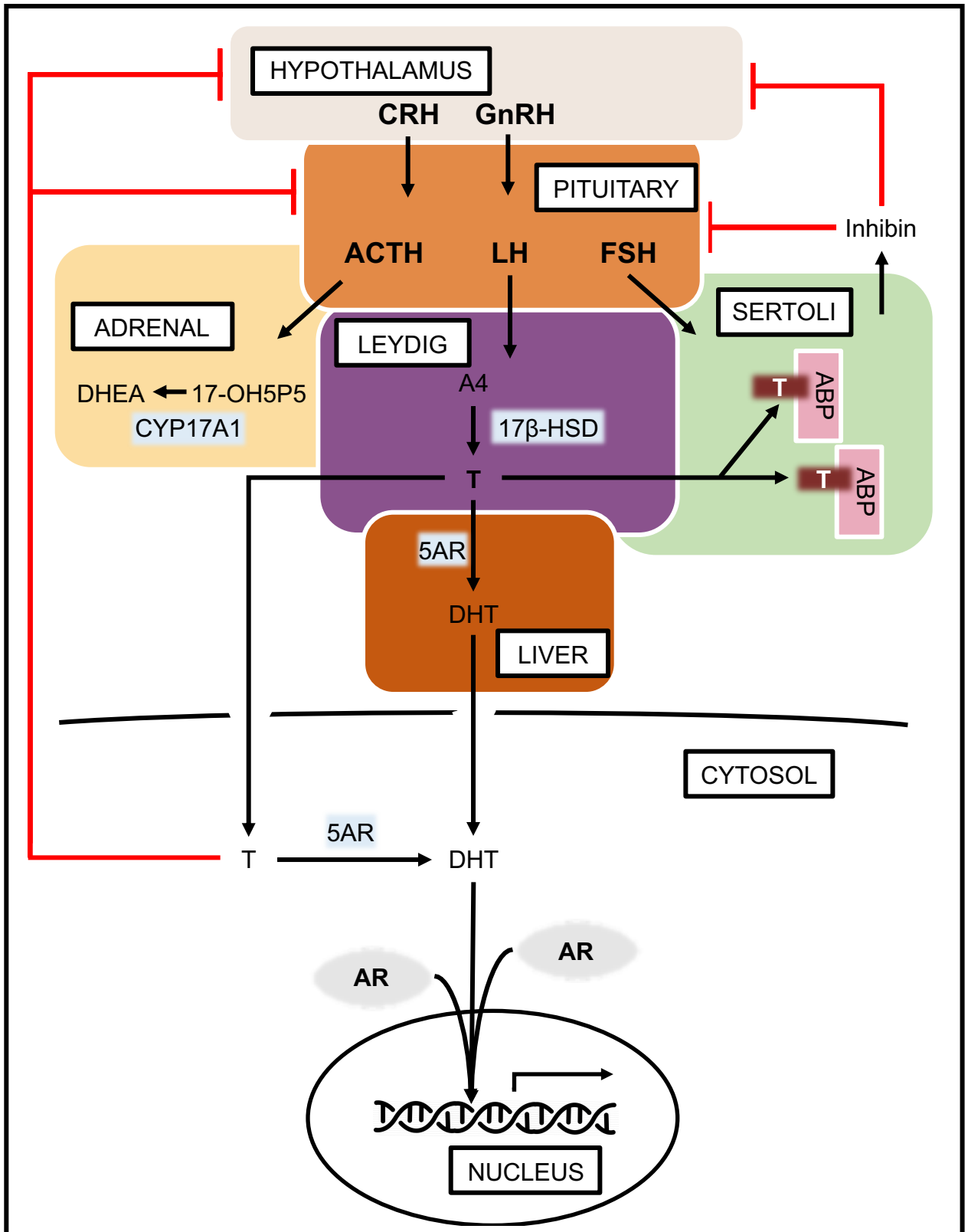

Figure 2 The Hypothalamic-Pituitary-Gonadal-Adrenal (HPGA) axis.
The HPGA axis consists of hypothalamic-pituitary hormones. Androgen synthesis consists of androgen metabolites, steroidogenic enzymes. and the androgen receptor (AR).

In addition to its regulatory role in spermatogenesis, T also modulates muscle and bone mass, secondary sex characteristics, sexual reproductive behavior, and sexual differentiation in men (Bhasin and Jameson, 2005). Notably, androgens are involved throughout the entirety of prostate formation and growth from early embryology to development of prostatic hyperplasia. During early embryogenesis, T acts to virilize the urogenital tract by inducing development of the wolffian ducts, vas differentia, epididymides, and seminal vesicles. As expression of 5α-reductase (5AR) begins in embryonic tissues, DHT is synthesized **(Figure 2)**, which stimulates development of the urethra and prostate and contributes to the formation of the penis and scrotum (Wilson, 2011). During puberty, androgens continue to regulate the final phases of development including branching morphogenesis and maturation (Prins and Putz, 2008).

In 1941 Huggins and Hodges were first to reveal the significance of androgen signaling in PCa by demonstrating that orchiectomy significantly reduces tumor regression (Huggins and Hodges, 1972). Later studies confirmed that DHT induced cell proliferation in PCa cells that was mediated by AR activation**.** AR is a nuclear hormone receptor with high affinity to DHT that when activated, recognizes androgen-response elements (AREs) in promoter regions of genes throughout the genome. **(Figure 2).** (Claessens et al., 2001; Yang et al., 2005). These and more studies led to the development of numerous variations of androgen deprivation therapies (ADT) which remain the mainstay of treatment for advanced disease. Human clinical trial evidence using the dual 5α-Reductase inhibitor dutasteride and the type 2 5α-Reductase inhibitor finasteride both decreased risk of PCa development by ~24% over a 4–7-year duration (Andriole et al., 2010; Thompson et al., 2003), Although these studies contribute to our knowledge of androgens promoting PCa risk, the duration of intervention was brief and conducted in older men with high occurrence of small prostatic cancers. Therefore, it is unknown whether androgens are required for carcinogenesis but rather influence the progression and growth of pre-existing neoplasms. Overall, the relationship between serum androgens and PCa risk has largely been inconsistent (Eaton et al., 1999; Gann et al., 1996a; Hyde et al., 2012; Shaneyfelt et al., 2000). The most

convincing evidence that androgens promote PCa comes from the NBL rat strain administered with T-propionate containing cholesterol pellets for 6–8-week intervals that caused visible prostate adenocarcinomas in 19% of mice (Bosland, 2013).

E. The Free Hormone Hypothesis and Megalin-mediated import

In human serum, the majority of steroid hormones, including 25D (~88%) (Powe et al., 2011) and T (~70%) (Li et al., 2016), do not circulate freely, but are bound to their serum globulins, the vitD binding protein (DBP) and sex-hormone binding globulin (SHBG), respectively, with high affinity (Fraser, 1983; Murphy, 1968). These carrier proteins function to regulate hormone transport, tissue distribution, metabolism, and bioactivity (Goldman et al., 2017). Globulin-bound hormones are sequestered extracellularly, rendering them unavailable to tissues, allowing only unbound fractions cellular access. These concepts were coined by Carl Mendel as The Free Hormone Hypothesis (FHH) (Mendel, 1989a) **(Figure 3)** which has been argued extensively (Goldman et al., 2017; Hammes et al., 2005; Nykjaer et al., 1999; Richards et al., 2017), with some groups claiming that both free and unbound entry mechanisms may exist in the same cell type. (Adams, 2005; Hammes et al., 2005)

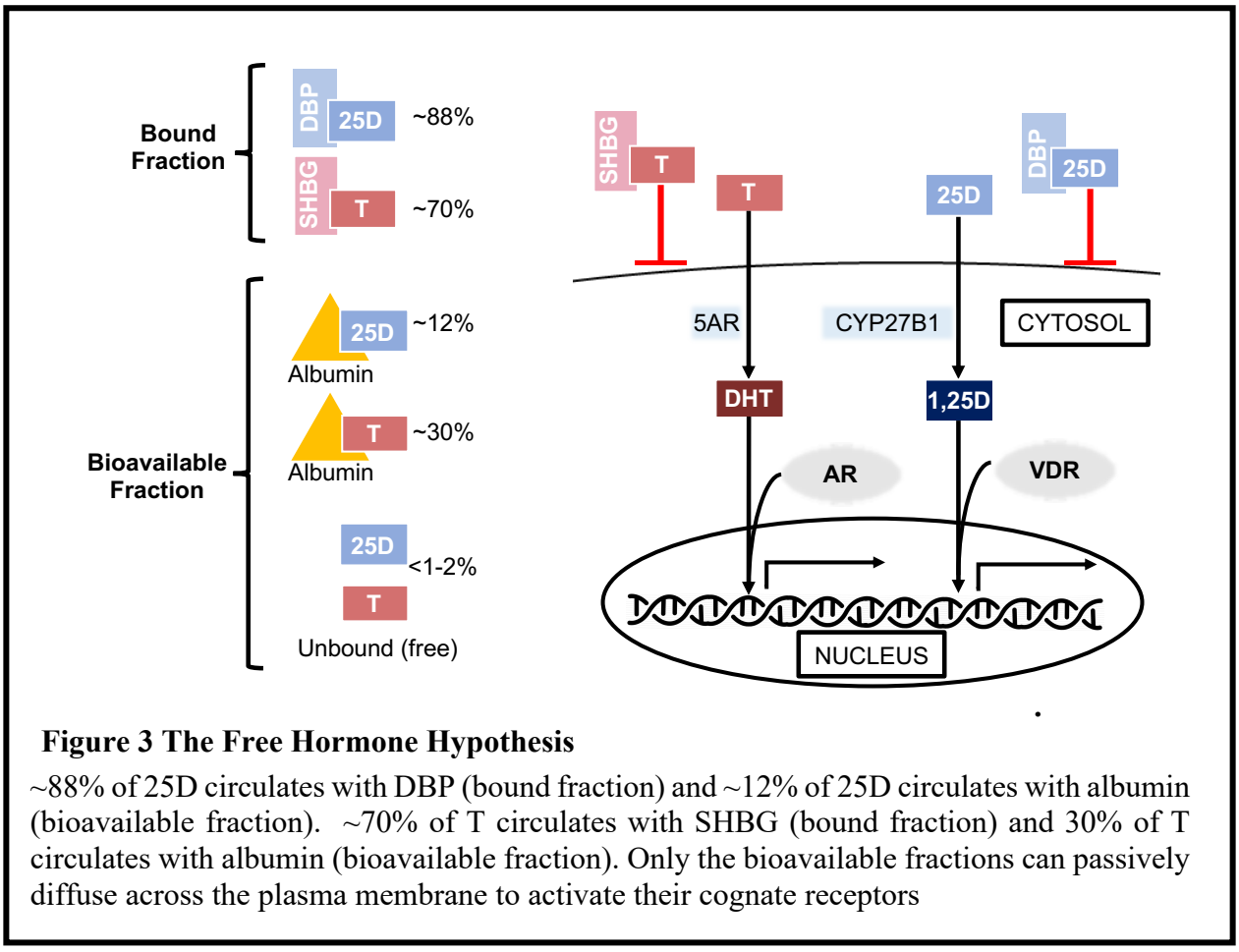

Figure 3 The Free Hormone Hypothesis

~88% of 25D circulates with DBP (bound fraction) and ~12% of 25D circulates with albumin (bioavailable fraction). ~70% of T circulates with SHBG (bound fraction) and 30% of T circulates with albumin (bioavailable fraction). Only the bioavailable fractions can passively diffuse across the plasma membrane to activate their cognate receptors

Megalin, encoded by the gene *LRP2*, is a multiligand 600 kilodalton type 1 transmembrane receptor capable of binding and internalizing a myriad of carrier proteins such as albumin, myoglobin, hemoglobin, lactoferrin, selenoprotein P, and sex-hormone binding globulin (SHBG). Megalin also binds vitamin carrier proteins like transcobalamin, retinol-binding protein, folate-binding protein, and vitamin D-binding protein (DBP). The amino-terminal contains four cysteine-rich ligand binding domains interspaced between 17 EGF-like repeats and eight YWTD motifs involved in pH-dependent dissociation of ligands. The cytoplasmic tail contains two NPXY motifs and a VENQNY motif believed to mediate signal transduction. Megalin is primarily expressed in polarized epithelial cells of various tissues including renal proximal tubules, epididymis, intestine, brain, and thyroid and may colocalize and function with its endocytic binding partner Cubilin, although MEGALIN has the capacity to function on its own. (Christensen and Birn, 2002)

The notion that both free and unbound cellular entry mechanisms exist simultaneously is conceivable. Megalin is capable of globulin-bound steroid hormone cellular import. Studies have demonstrated SHBG-bound T is internalized in human prostate cancer cells (Li et al., 2016) and in rat yolk sac in a MEGALIN-mediated manner (Hammes et al., 2005) Additionally, >90% of MEGALIN-deficient mice die perinatally, but the remaining offspring display impaired descent of the testes into the scrotum (Hammes et al., 2005), develop extreme proteinuria for which DBP is the main species (Leheste et al., 2003), and have reduced levels of serum 25D while exhibiting drastic bone defects (Nykjaer et al., 1999). In humans, dysfunctional MEGALIN causes Donnai-Barrow syndrome, characterized by craniofacial anomalies, deafness, myopia, and increased urinary DBP (Storm et al., 2013). These compelling studies reveal MEGALIN's role to regulate SHBG-bound T uptake and bioactivation in reproductive tissues in addition to its well-described renal function to resorb DBP-bound 25D from the glomerular filtrate.

Our lab recently confirmed that circulating levels of 1,25D and prostatic levels of 1,25D are not concordant (Richards et al., 2017), indicating passive diffusion is not the only mechanism

of prostatic vitD uptake. Since nearly all of 25D is bound and sequestered in the serum by DBP, our data support a mechanism by which this bound vitD is imported into the prostate.

We also observed that prostate epithelium expresses MEGALIN protein and that prostatic expression of *LRP2* negatively correlated with prostatic 25D in AA men only and positively correlated with percent West African Ancestry (%WAA) **(Figure 4)** (Richards et al., 2017). The overarching conclusions of these observations suggest that the FHH may not apply in all situations and infer an ancestry-specific compensatory mechanism of vitD transport into prostate epithelium when systemic levels are deficient.

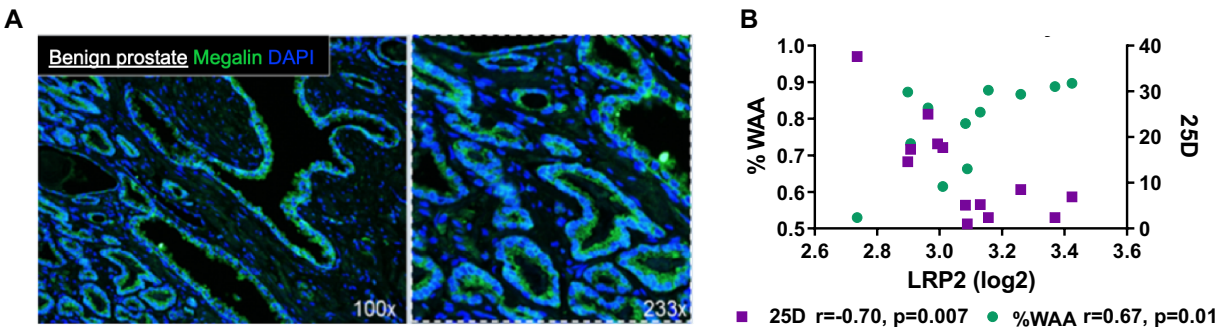

Figure 4 Megalin protein expression and *LRP2* relationships with prostatic 25D and West African Ancestry

A, Megalin protein (green) was detected on the luminal compartment of benign prostate epithelium. Nuclei counterstained with dapi (blue). **B**, *LRP2* inversely correlates with prostatic 25D and positively correlates with West African Ancestry. (Richards et al., 2017)

F. Hypothesis and Goals of the Thesis

AA men are more prone to both vitD deficiency and developing early-onset PCa. VitD is well-established as a potentially anti-cancer agent and androgens are known drivers of PCa progression. However, a paucity of knowledge currently exists regarding the interplay between circulating and intraprostatic steroid hormone levels, prostatic hormonal import, and PCa disparities in the context of vitD deficiency. Therefore, we hypothesized that MEGALIN mediates prostatic 25D and T import and that MEGALIN is regulated by steroid hormones. Additionally, we hypothesize that MEGALIN expression positively correlates with %WAA and is increased in the setting of vitamin D deficiency. To address this, we performed a mechanistic examination of steroid hormone transport and receptor activity in benign and cancer prostatic epithelial cells (PrEs) and tissue explants, determined relationships between vitD and androgen metabolites in serum and prostate tissues from AA and EA patient specimens, and examined MEGALIN expression in benign and cancer prostate tissues and cells.

CHAPTER II: RESULTS

A. *LRP2*/MEGALIN expression varies in prostate epithelial cells

Megalin function has been well-characterized in the kidney (Chapron et al., 2018) and its expression has been reported in human prostate epithelium by immunofluorescence (Richards et al., 2017; Ternes and Rowling, 2013). To determine *LRP2* (MEGALIN gene) and MEGALIN expression in our prostate cell models, we performed RT-qPCR and western blotting techniques to first detect *LRP2* mRNA and protein expression. Using human embryonic kidney cells (HEK 293) as a positive control, we assessed *LRP2* **(Figure 5A)** gene and MEGALIN protein **(Figure 5B-C)** expression which varied across prostate cell lines including PrEs derived from two AA radical prostatectomy patients, the immortalized prostate epithelial cell line 957E-hTERT, and the immortalized and transformed prostate cancer cell lines LAPC4 and 22Rv1. Given MEGALIN's primary renal role, HEK293 cells exhibited the highest levels of MEGALIN gene and protein expression followed by 22Rv1 cells that exhibited the highest expression of *LRP2* mRNA and MEGALIN protein compared to all other prostate-derived cell lines. Surprisingly, the remaining cell lines expressed lower levels of *LRP2* and MEGALIN, with LAPC4 expressing the lowest.

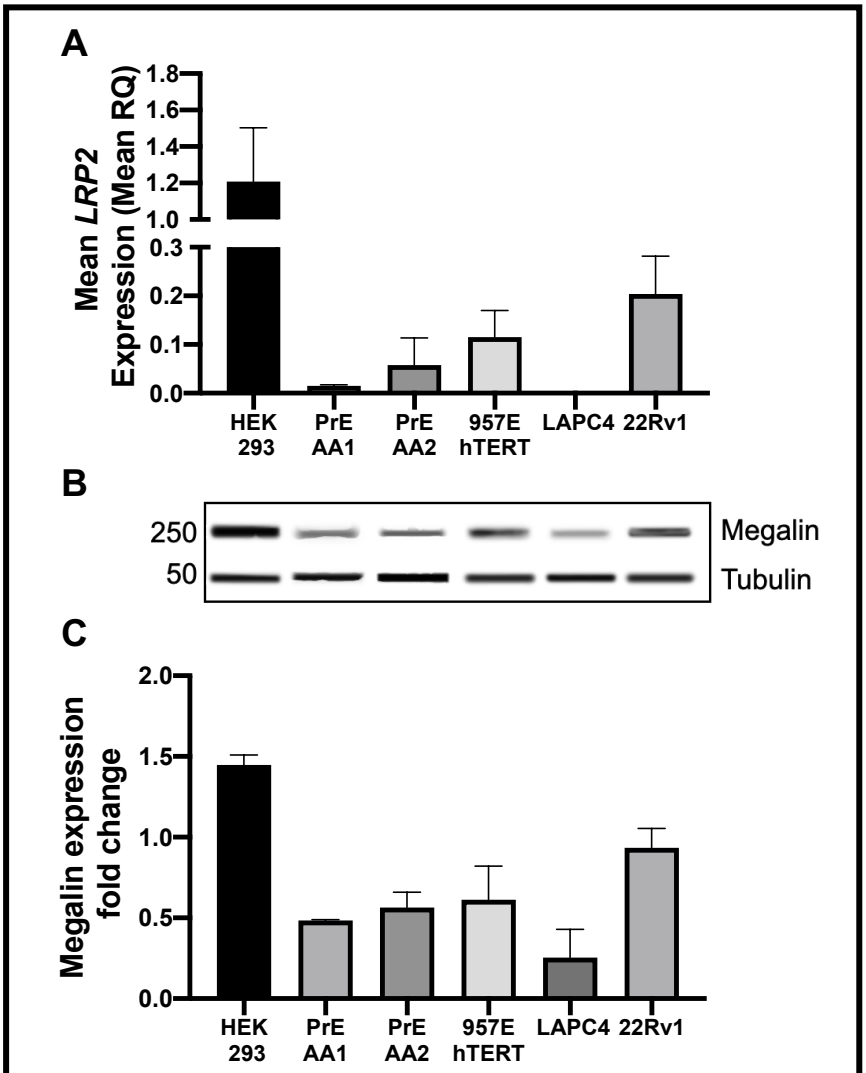

Figure 5 *LRP2* and MEGALIN expression in benign and cancer prostate epithelial cells.

A, RT-qPCR for *LRP2* in PrE AA1-2, 957E-hTERT, LAPC4, and 22Rv1. Western blot expression **(B)** and quantification **(C)** of MEGALIN protein in PrE AA1-2, 957E-hTERT, LAPC4, and 22Rv1. HEK 293 shown as a positive control. Relative gene expression shown as relative quantity (RQ) normalized to *HPRT1*. Graphs represent mean ± SD of 1 experiment with 2 replicates per experiment.

B. Megalin facilitates transport of SHBG-bound T into prostate cancer epithelial cells

As discussed in the introduction, Megalin binds and internalizes SHBG, the serum carrier of T. There is one report of SHBG-bound T import in PCa cells (Li et al., 2016) and MEGALIN was shown to import SHBG-bound T in mouse hippocampal cells (Caldwell et al., 2007). To determine if this mechanism extends to the prostate, the androgen-responsive LAPC4 and 22Rv1 prostate cancer cell lines were used to examine SHBG-bound T import and AR activation. PrEs were not used because they exhibit a basal and AR-negative phenotype. SHBG purified from human serum was labeled with an amine-reactive orange, fluorescent Alexa Fluor 555 dye (SHBG-555) and added to LAPC4 and 22Rv1 cells in the presence and absence of T. T-treated LAPC4 and 22Rv1 cells appeared vesicular, indicating active uptake of SHBG-bound T. Co-treatment with receptor associated protein (RAP), an inhibitor of endocytic activity of the MEGALIN receptor (Chapron et al., 2018) diminished SHBG-555 internalization, suggesting MEGALIN-mediated internalization of SHBG-bound T **(Figure 6A-B).**

To determine if internalized SHBG-bound T increase AR activity, genomic activation of AR was examined by several endpoints. LAPC4 and 22Rv1 cells treated with 50 nM T +/- 250 nM SHBG showed increased KLK3 gene expression, a known target of AR which was inhibited by RAP **(Figure 7A)**. LAPC4 and 22Rv1 cells transfected with a luciferase reporter driven by two promoter-derived AREs $(ARE)_2$ (Zhang et al., 2000) and treated with 50 nM T +/- 250 nM SHBG showed increased luciferase activity that was also inhibited by RAP **(Figure 7B)**. In 22Rv1 cells treated with T/SHBG, a significant increase in AR protein was observed while cells treated with T alone showed increased nuclear AR but was not significant. From this, we conclude that LAPC4 and 22Rv1 cells can internalize SHBG-bound T via MEGALIN binding.

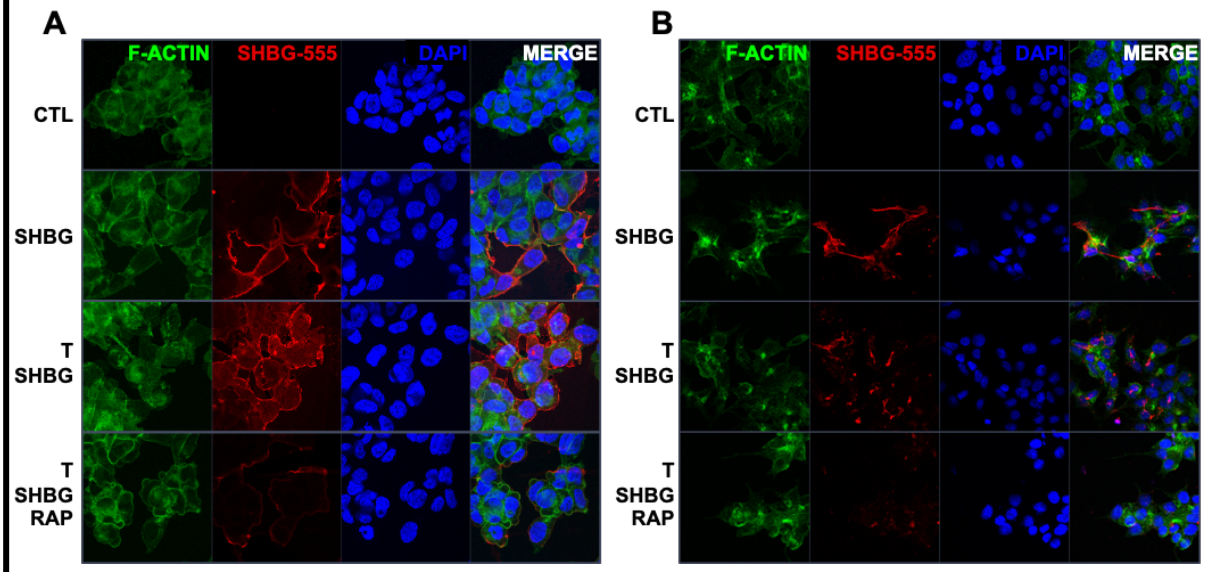

Figure 6 SHBG-bound T is imported in LAPC4 and 22Rv1 PCa cells

A, SHBG-555 (red), DAPI (blue), and F-actin (green) imaged after 4 hr incubation in LAPC4. **B**, SHBG-555 (red), DAPI (blue), and F-actin (green) imaged after 4 hr incubation in 22Rv1 (63X magnification)

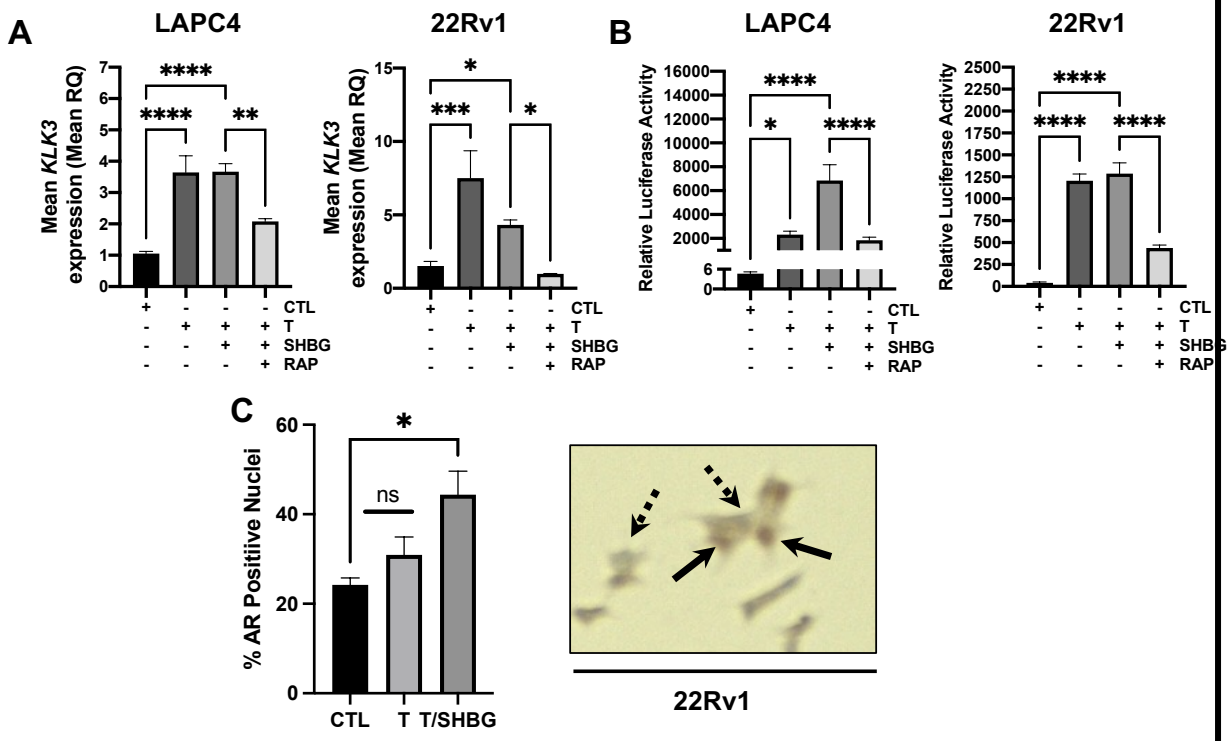

Figure 7 SHBG-bound T is imported and active in LAPC4 and 22Rv1 PCa cells.

A, RT-qPCR for *KLK3* in LAPC4 (left) and 22RV1 (right) cells 16 hr after treatment with 50nM T in the presence and absence of 250nM SHBG. Relative gene expression shown as relative quantity (RQ) normalized to *HPRT1*. **B**, $(ARE)_2$ promoter activity in LAPC4 (left) and 22RV1 (right) cells 24 hr after treatment with 50nM T in the presence and absence of 250nM SHBG. **C,** Detection of nuclear AR protein 22Rv1 cells 4 hr after treatment with 50nM T in the presence and absence of 250 nM SHBG. Arrows: solid indicate AR positive nuclei, dashed indicate AR negative nuclei. N = not significant P value determined using unpaired t test indicated with * $P < 0.05$; ** $P < 0.002$; *** $P < 0.0002$; **** $P < 0.0001$). Graph represents mean ± SEM of 2 individual experiments with 2 replicates per experiment.

C. DBP-bound 25D is internalized and active in prostate epithelial cells

VitD status by serum levels of 25D is the metric most often used in epidemiologic studies of vitD and cancer, whereas tissue concentrations of vitD have not been well-studied (Chun et al., 2014). As outlined in **Chapter I**, our lab previously reported that AAs had higher levels of 1,25D in the prostate despite being deficient for 25D in circulation. In contrast to the FHH, this finding indicates that 25D may be imported and locally converted to 1,25D, and indicate that systemic levels of 25D do not accurately depict one's vitD status. Megalin renal role to internalize DBP has been well characterized. (Chapron et al., 2018). To determine if this mechanism extends to the prostate, PrEs derived from an AA RP patient (PrE -AA1) and 957E-hTERT cells were used to examine DBP-bound 25D import and VDR activity in vitro. DBP purified from human serum was labeled with an amine-reactive green, fluorescent Alexa Fluor 488 dye (DBP-488) and added to PrE-AA1 cells in the presence and absence of 25D. The internalization of DBP-488 in the presence of 25D appeared vesicular, indicating active uptake of DBP-bound 25D. Co-treatment with RAP diminished DBP-488 internalization, suggesting MEGALIN-mediated internalization of DBP-bound 25D **(Figure 8A).**

In humans the majority of systemic 25D (~88%) (Powe et al., 2011) circulates bound to DBP with high affinity (K_d=10nM) and is present in 30-fold excess compared to 25D (Mc Leod et al., 1989). To determine if internalized DBP-bound 25D results in increased VDR activity, genomic activation of VDR was examined. PrE-AA1 organoids were grown for 7 days and treated with physiologically relevant concentrations of 25D (Pagana and Pagana, 2012) ranging from 10nM to 100nM in the presence and absence of +/- 0.02ug/uL DBP (the amount of DBP present in 5% human serum) showed increased CYP24A1 gene expression, a classic target of VDR (Jones et al., 2012), across all 25D treatments**.** The treatment condition that most closely depicted the human DBP/25D molar ratio was the 10nM 25D + DBP condition. It is unclear why the CYP24A1 induction observed was mostly lower in the +DBP conditions. DBP concentrations in human serum are within micromolar ranges whereas 25D are in nanomolar. We used 5% of human DBP

concentrations so this may be a result of passively diffused 25D-mediated CYP24A1 induction Alternatively, the bound 25D could create an extracellular concentration gradient to efficiently provide continuous cellular passive diffusion of hormones. **(Figure 8B)**.

To further determine if imported 10nM 25D activates the VDR, 957EhTERT cells were transiently transduced with a GFP-labeled VDR and treated with 25D + 0.02ug/uL DBP. Nuclear translocation of VDR was observed after co-incubation with 25D-DBP, indicating 25D-DBP internalization and subsequent VDR activation **(Figure 8C).** From this we conclude that 10nM 25D is bound, internalized, and biologically active in prostate epithelial cells.

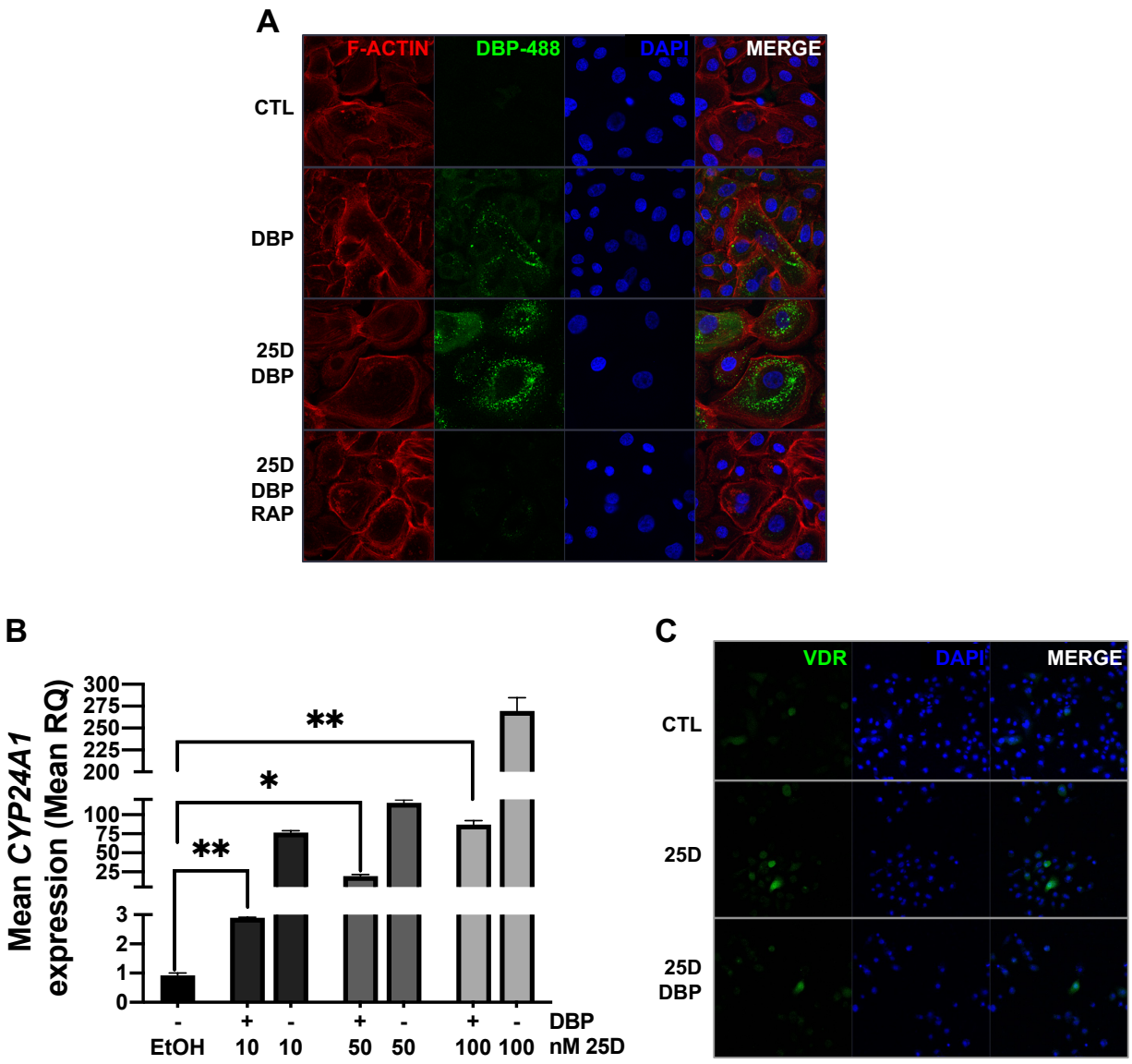

Figure 8 DBP-bound 25D is imported and active in prostate epithelial cells.

A, DBP-488 (green) DAPI (blue) and F-actin (red) imaged after 4 hr incubation in PrE-AA1 cells (63X magnification). **B,** RT-qPCR for *CYP24A1* in PrE-AA1 organoids 24 hr after treatment with 25D in the presence and absence 0.02ug/uL DBP. C, VDR-GFP (green) and DAPI (blue) imaged after 4-hour incubation in 957-EhTERT cells. Relative gene expression shown as relative quantity (RQ) normalized to *HPRT1*. P value determined using unpaired tests indicated with * $P < 0.05$; ** $P < 0.002$. Graph represents mean ± SD of 1 individual experiment with 2 replicates per experiment.

D. *LRP2*/MEGALIN expression is inhibited by vitD and androgen in human and murine prostate cells

Our recent study found an inverse correlation between prostatic 25D and *LRP2* expression only in AA men **(Figure 4)** (Richards et al., 2017). However, there were very few white men with low 25D, which may have biased the results. The ability of 25D and T, separately, to regulate *LRP2* expression *in vitro* was assessed in PrEs derived from an AA patient (PrE-AA2), 957E-hTERT cells, and 22Rv1 cells. 25D and T decreased *LRP2* mRNA expression in PrE-AA2 and 22Rv1 cells (**Figure 9A**). The *LRP2* promoter was found to have 6 VDRE half-sites and 5 ARE half-sites (**Figure 10)**, was cloned into a luciferase reporter plasmid **(Figure 9B)** and transfected into 957E-hTERT and 22Rv1 cells, which showed decreased promoter activity after treatment with 10nM 25D and T (**Figure 9C**). Megalin expression was also inhibited in 957E-hTERT and 22Rv1 cells treated with 1,25D and the synthetic androgen analog, R1881 **(Figure 9D)**. These data indicate that *LRP2* and MEGALIN expression may be negatively regulated by vitD and androgen, which corroborate our correlations between prostatic 25D and *LRP2* in patient material **(Figure 4)**

Using single-cell RNA sequencing, Karthaus et al studied the gene expression profile of mouse prostates during a complete castration/regeneration cycle. (Karthaus et al., 2020). This procedure, illustrated in **Figure 11A,** involves castration to allow prostate involution and T add-back at day 28 to allow regeneration. The luminal epithelial subpopulations of these animals showed *LRP2* expression increases after castration and involution, then decreases after T re-substitution and prostate regeneration; evidence further implicating how androgens may be regulating the expression of *LRP2* **(Figure 11B).**

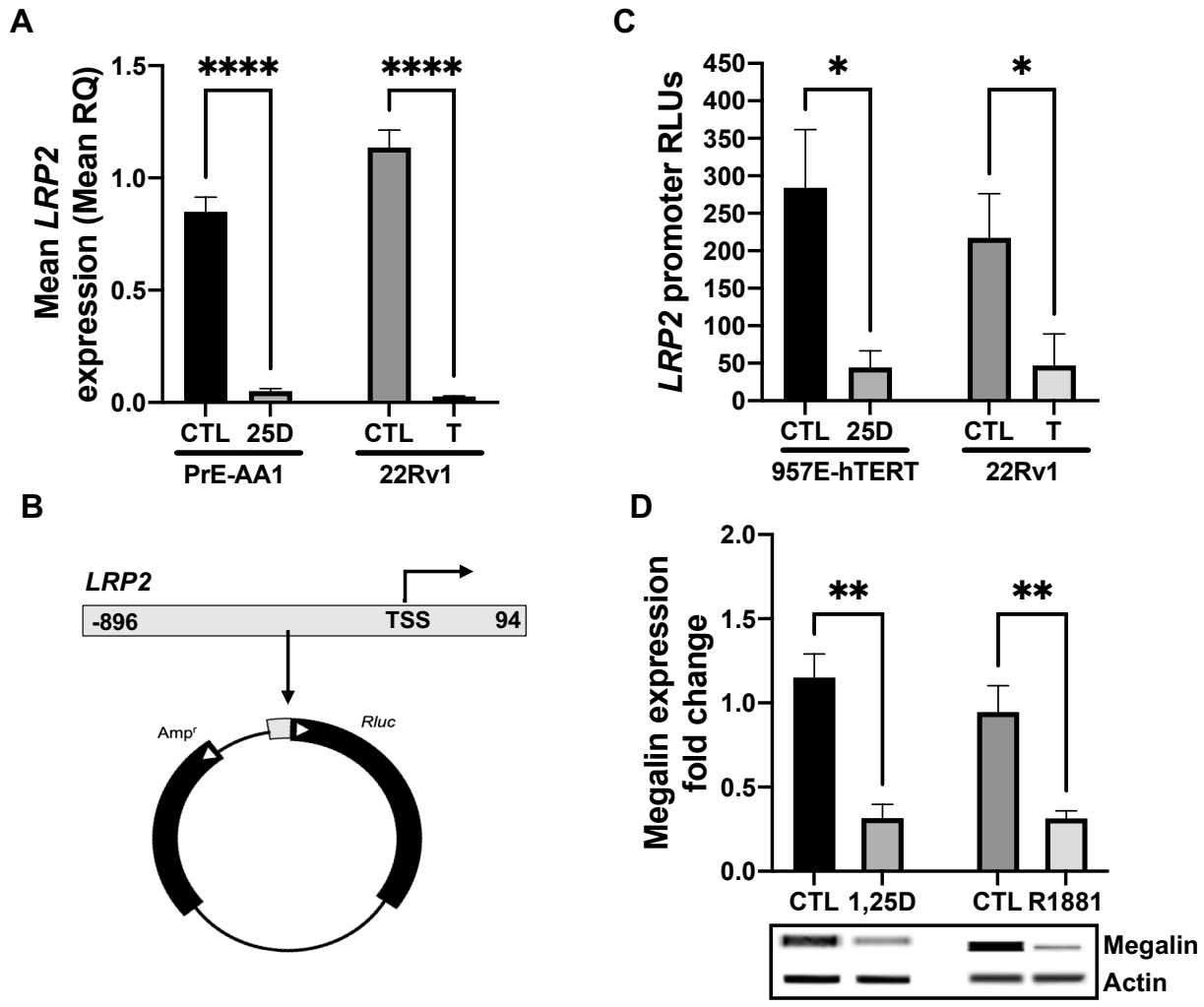

Figure 9 Hormonal regulation of *LRP2*/MEGALIN expression.

A, RT-qPCR for *LRP2* 16 hr after treatment with 10nM 25D in PrE-AA2 cells (left) or with 10nM T in 22Rv1 cells (right). Relative gene expression shown as relative quantity (RQ) normalized to *HPRT1* **B**, Schematic of the VDRE/ARE containing *LRP2* promoter-driven luciferase reporter (TSS, transcription start site). **C,** *LRP2* promoter activity in 957E-hTERT cells treated with 10nM 25D (left) or with 10nM T in 22Rv1 cells (right) normalized to *Photinus* luciferase activity. RLU = Relative Luciferase Units**. D,** Detection of MEGALIN protein expression 72 hours after treatment with 50nM 1,25D in 957E-hTERT cells (left) or with 50nM R1881 in 22Rv1 cells (right).

CCTGTATCCTATCAGGGGAAACCAAGA
TCCGAAGGAGCCTAGAGAGATCAGGCCAGGAATGAAGGTCACAGATCTCCATCCA
GCAGGCTCTTTACTCCTTCCCTTCCCTACCCCCGCCTCTTTCCCTTTCTTTCTCTTTC
CTTCATTTTTACTTGTCTGGTTGTGCTTTCCTTTTCCATTTCTTATTTTTTCTTTCTCT
TCTCTATTCTTGACTTTCCTCCTCGTCCATTTCTTTCCATTGCTCATTTAGTAAAACTG
CGTCCGTGCAGATTTCCCCGTGAGCCGCCTCGCCGGCTTCTGTGCGGGGCAAACC
CTGCGGAGAGGCGAGGGCAGCGCGTGTGCACGTGTGAGTGTGCCTGTGAGAGTGT
GCGCACAGGAGTGTGCTCTTGTATGCACGCGTAAAGGGGACTGTGTATGTCGGCGT
TGAAGTGTGACTGTGGAATGTGCGCGCGTGTGTGACTGGCGTGTATGAGTGAATCT
GTGTCAGTTGGTGTGACACTTAGGAGTGCATGCGCCTGTATGAGTGCGTGTGTGAG
TGTGCACACGCCTGTGTGAGTGTCCCTGTGTGAGCACGCGTGTGTTCGAGTGCCC
ACTCCTCCCCGCGCTGCAAAGTGCAGGGGGCGGGCCGGGCGGCAGGGGGCCTGC
CCGCTGGATTCCCGCATGCTTGTTCCGGGGCGGTCGGGTCTAAAGGGCTTTATGCA
CTGTCTGGAGGGTGGGGACTGGCGCGGGTAGAAAACGGGATGCCTCGGfagGCGTG
GGGGCAGGCTTTTGGCCACTAGGACTGGCGGAGGTGCAGACCTAAAGGAGCGTTC
GCTAGCAGAGGCGCTGCCGGTGCGGTGTGCTACGCGCGCCCACCTCCCGGGGAA
GGAACGGCGAGGCCGGGGACCGTCGCGGAGATGGATCGCGGGCCGGCAGCAGT
GGCGTGCACGCTGCTCCTGGCTCTCGTCGCCTGCCTAGCGCCGGCCAGTGGCCAAG
GTAAGAGCCCAGCCAGAG

Figure 10 The Human *LRP2* promoter sequence

The *LRP2* promoter contains 6 VDRE half sites (purple, underlined, bold), 5 ARE half sites (red, underlined, bold**),** and 2 ERE (estrogen receptor element) half sites (green, underlined, bold) upstream of the **t**ranscription start site (ATG, underlined).One ERE and one VDRE overlap, indicated with an arrow (top of page)

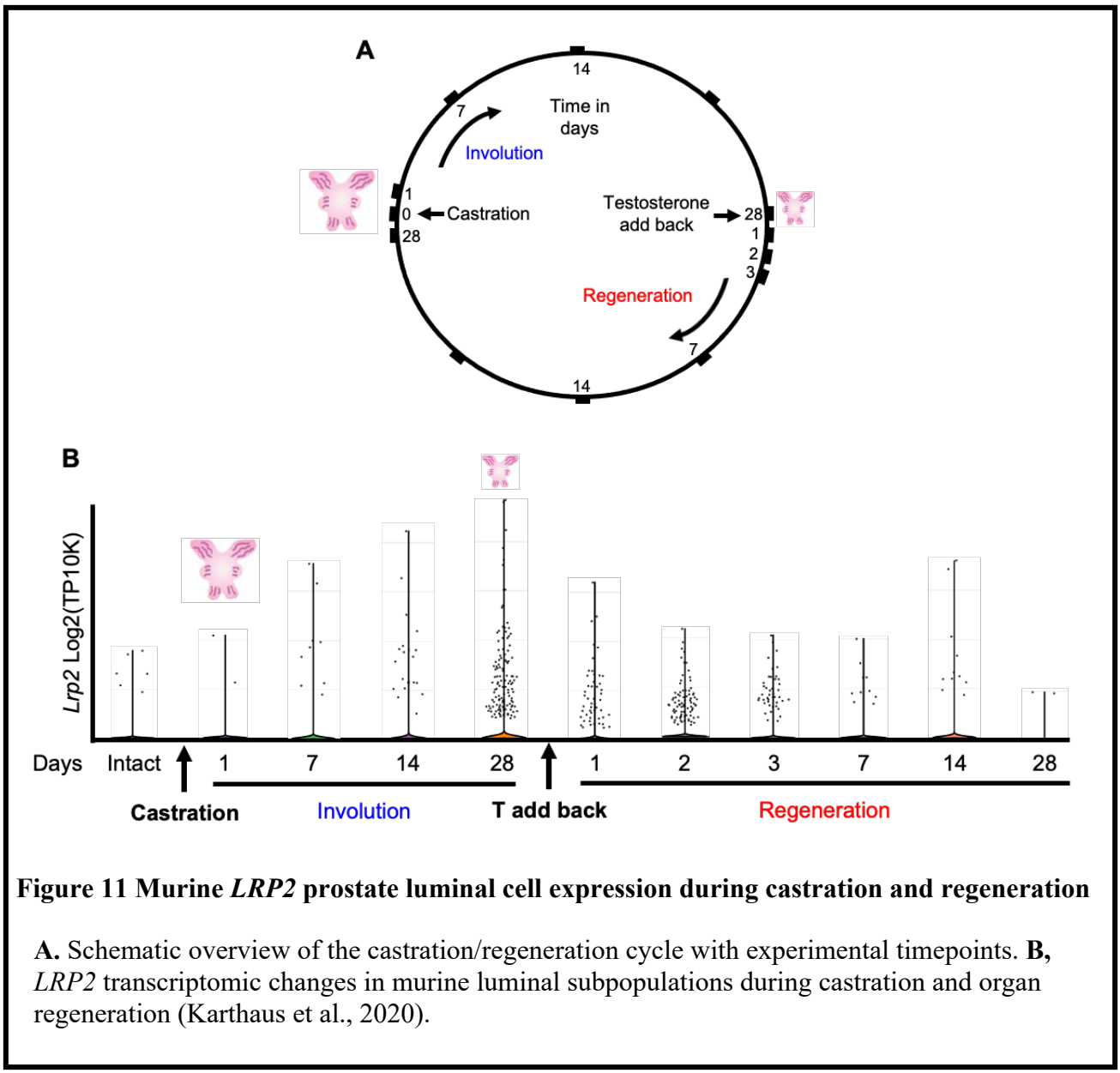

Figure 11 Murine *LRP2* prostate luminal cell expression during castration and regeneration

A. Schematic overview of the castration/regeneration cycle with experimental timepoints. **B,** *LRP2* transcriptomic changes in murine luminal subpopulations during castration and organ regeneration (Karthaus et al., 2020).

E. Prostate tissue slice explants respond to SHBG-bound T and DBP-bound 25D

To determine if prostate tissue can endocytose and respond to globulin-bound 25D and T, fresh benign human prostate tissue was cultured ex vivo as precision cut 300-micron sections. **(Figure 12A)**. The tissue slice explants express all components of the androgen and vitD activation and response pathways; CYP27B1 (vitD 25-hydroxylase), VDR, *LRP2*, SRD5A (T to DHT conversion), and AR. The explants retain complex biological interactions among glandular epithelium and the surrounding stromal compartments and currently are considered to be the most realistic preclinical prostate models (Maund et al., 2014). **(Figure 12B)**. Tissue slices treated with 50nM T or with 50nM T pre-incubated with 250 nM SHBG showed induction of KLK3 expression **(Figure 12C).** CYP24A1 was robustly induced in the slices by 50nM 25D alone or with 50nM 25D preincubated with 0.02ug/uL DBP **(Figure 12D).** *LRP2* expression was decreased in tissue slices treated with 50nM T and 25D alone **(Figure 12E),** consistent with our PrE and 22Rv1 cells **(Figure 8A)**. These findings strongly support the internalization and activation of SHBG-T and DBP-bound 25D in human prostate tissue and demonstrate the biological validity of hormone-mediated negative-feedback on *LRP2*.

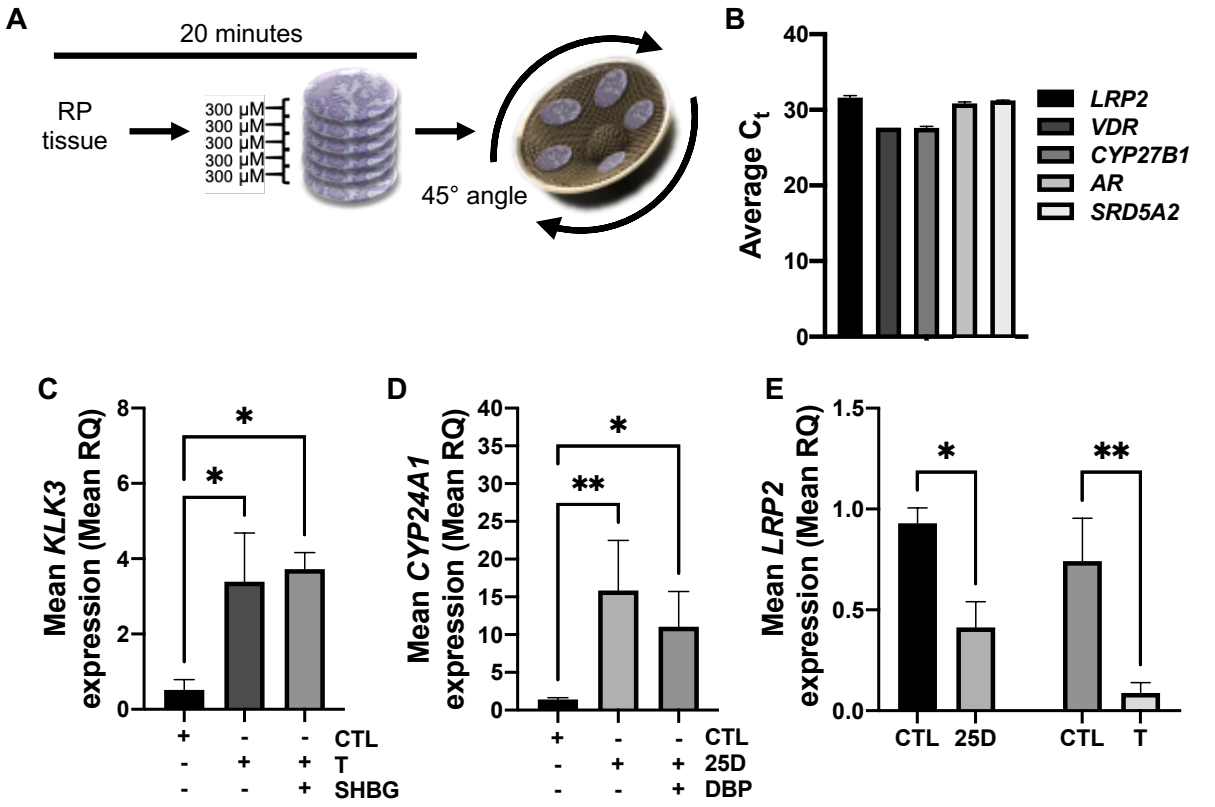

Figure 12 Patient-derived prostate tissue slices actively import and respond to SHBG-bound T and DBP-bound 25D.

A, *Ex-vivo* prostate tissue slice workflow. **B,** RT-qPCR for *CYP27B1*, *VDR*, *LRP2, SRD5A2* and *AR* mRNA in prostate tissue slices. **C**, RT-qPCR for *KLK3* in prostate tissue slices 16hr after treatment with 50nM T in the presence and absence of 250nM SHBG. **D,** RT-qPCR for *CYP24A1* in prostate tissue slices 16 hr after treatment with 50nM 25D in the presence and absence of 0.02 µg/µL DBP. **E**, RT-qPCR for *LRP2* in prostate tissue slices 16 hr after treatment with 50nM T and 50nM 25D. Relative gene expression shown as relative quantity (RQ) normalized to *HPRT1.* P value determined using unpaired tests indicated with * $P < 0.05$; ** $P < 0.002$. Graphs represent mean ± SEM of two experiments with 2 replicates per experiment.

F. Intraprostatic DHT is higher in African American men and inversely correlates with vitamin D status.

Our in vitro data established that *LRP2* is negatively regulated by 25D and T and that globulin-bound T and 25D can enter prostate cells and tissues. Translating this to patients, this suggests that vitD deficiency, and low serum T levels may upregulate MEGALIN, resulting in increased import of both 25D-DBP and SHBG-T **(Figure 13E).** Indeed, our prior study found this relationship with vitD in patient specimens, as AAs had significantly lower serum 25D yet significantly higher intraprostatic 1,25D compared to EAs. We quantified T and DHT in serum and prostate tissue from a cohort of prostate cancer patients for whom we have previously measured vitamin D metabolites (Richards et al., 2017). Pre-surgical serum and benign areas of radical prostatectomy specimens were used for all hormone measurements. In the serum, T was the more abundant metabolite while DHT was more abundant in the tissue (**Figure 13A**). A few patients had notably high prostatic T with low DHT, suggesting they may have been on 5α-Reductase inhibitors at the time of surgery, although this was not recorded in the annotation. Although this cohort of AA men had lower serum T, they had significantly higher levels of intraprostatic DHT compared to EA men (**Figure 13B-C**).

Correlations between androgen and vitD metabolite concentrations were analyzed to determine if they support our hypothesis that vitamin D deficiency increases prostatic androgen levels. Intra-prostatic levels of DHT significantly positively correlated with tissue 25D and 1,25D in AAs **(Figure 14A-B).** Serum 25D and intra-prostatic DHT had a significant inverse correlation in the study population as a whole (**Figure 13D**). However, this correlation was not observed when the population was analyzed by ancestry and may reflect the lack of vitD deficient EAs and replete AAs in the study population. Serum 1,25D did not correlate with intra-prostatic DHT **(Figure 14C).** We previously reported that *LRP2* was inversely correlated with intra-prostatic 25D in AAs, but no association of *LRP2* expression with intra-prostatic DHT was observed **(Figure 14D).** Intra-

prostatic DHT did positively correlate with VDR expression in EAs and in the study population as a whole **(Figure 14E).**

Overall, the inverse correlation of serum 25D with intra-prostatic DHT, the positive correlation of intra-prostatic 25D with DHT, and lower serum T suggests serum vitD deficiency and decreased systemic T could be a driver of higher prostate DHT levels via MEGALIN upregulation. Indeed, these correlations underscore ancestry-specific differences in intra-prostatic vitD and androgens and allude to possible inter-play between the two metabolites.

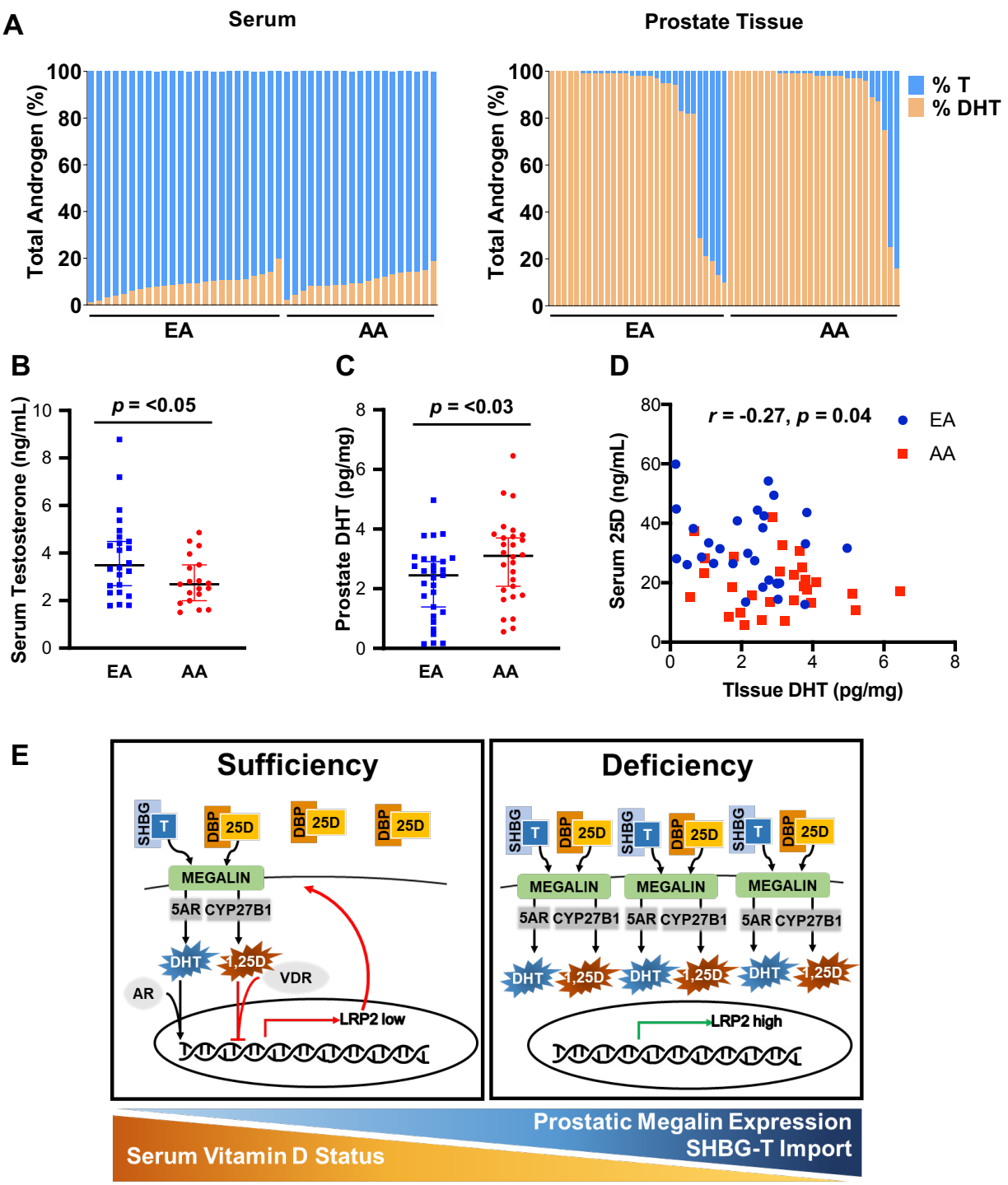

Figure 13 Ancestry-specific differences in androgen levels and relationships between DHT and 25D.

A, waterfall plots of the distribution of circulating serum (left) and prostate tissue (right) T and DHT; EA (n = 29) and AA (n = 28). **B,** comparison of serum levels of T (EA, n = 24; AA, n = 19) and, **C**, prostate tissue DHT (EA, n = 29; AA, n = 28) measured by uHPLC-MS-MS. Graphs represent mean with 95% confidence interval. P value determined using unpaired t test. **D**, correlation between serum 25D and prostate DHT in EA (n = 29) and AA (n = 28) men measured by uHPLC-MS-MS. Correlation determined using Spearman's rank. **E**, our proposed model of MEGALIN upregulation in the setting of vitamin D deficiency**.**

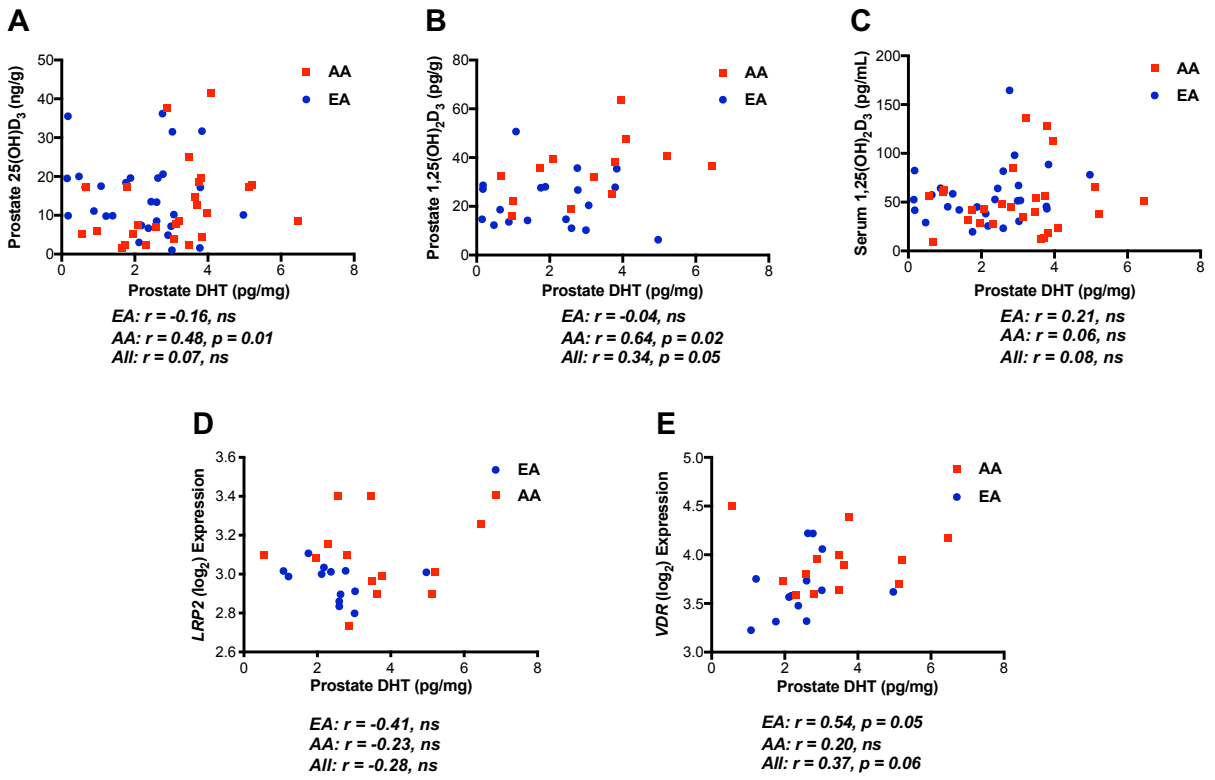

Figure 14 Relationships between the vitamin D and androgen signaling axes.

Relationships between prostate 25(OH)D and prostate DHT in EA (n = 29) and AA (n = 28) measured by uHPLC-MS-MS **(A)**. Relationships between prostate 1,25$(OH)_2$D and prostate DHT in EA (n = 29) and AA (n = 28) measured by uHPLC-MS-MS **(B)**. Relationships between serum 1,25$(OH)_2$D prostate DHT in EA (n = 29) and AA (n = 28) measured by uHPLC-MS-MS **(C)**. Relationships between *LRP2* expression and prostate DHT in EA (n = 13) and AA (n = 13) measured by uHPLC-MS-MS **(D)**. Relationships between *VDR* expression and prostate DHT in EA (n = 13) and AA (n = 13) measured by uHPLC-MS-MS **(E)**. Affymetrix array expression (reported as log) of *LRP2* and *VDR* collected by laser capture microdissection (LCM) from the benign epithelium of fresh frozen radical prostatectomy specimens **(D – E).** Correlations determined using Spearman's rank

G. Megalin expression is reduced in localized disease and increased in aggressive disease.

To determine if MEGALIN is altered in PCa, a tissue microarray (TMA) was used. The TMA was composed of 118 prostate biopsy cores from 29 patients (20 AA, 9 EA) with 2 cores from benign and cancer regions for each patient. Note that ancestry estimation and hormone levels were not determined for the patients represented on the TMA, thus correlation to %WAA and to hormones was not possible. Protein levels digitally were quantified by fluorescence intensity in the epithelial regions using the epithelial marker Pan-CK to demarcate the epithelium **(Figure 15A)**. The pathology and tissue mark-up of each epithelial region was determined by a board-certified pathologist. Megalin protein levels were significantly lower in the cancer tissue compared to benign in the overall cohort ($p=0.02$) and in AA group ($p = 0.0006$). Megalin did not significantly differ in EA patients, although EA may just require more patients to reach significance **(Figure 15B).** Overall, MEGALIN protein levels were lower in PCa.

Given that *LRP2* expression differed in cancer regions compared to benign tissue and is regulated by androgen, we analyzed *LRP2* mRNA expression in castration-resistant prostate cancer (CRPC), a progressive form of PCa that develops after surgical or chemical castration (first line ADT therapies). These treatments are designed to disrupt androgen signaling by prevention of androgen formation. Unfortunately, the effects of castration are permanent, and the disease becomes what is termed CRPC, characterized as aggressive androgen independent disease. This outcome occurs in most patients in response to first line ADTs. (Kregel et al., 2016). To determine if *LRP2* expression differs between different PCa disease states, we analyzed an independent RNA-Seq cohort from twenty-five prostate tumors and fifty-one metastases (Bhanvadia et al., 2018; Pflueger et al., 2011; Robinson et al., 2015). We examined *LRP2* RNA in four sample types. The first comparison was between localized tumors and metastases. The localized carcinoma measurements are from patient radical prostatectomy tissues before beginning ADT therapy, and the metastases are from patients with CPRC, some of which had received taxane, abiraterone, or enzalutamide (a second line ADT classified as a non-steroidal anti-androgen and AR antagonist,

which can also lead to resistance) (Kregel et al., 2016) Comparison of localized disease to metastatic tumors showed increased *LRP2* mRNA expression in metastatic progression (**Figure 15C**). In comparing metastases alone before and after abiraterone/enzalutamide treatment, *LRP2* expression decreased following abiraterone/enzalutamide treatment, suggesting loss of MEGALIN expression upon acquisition of abiraterone/enzalutamide-resistance. **(Figure 15D)**. To model this finding in in vitro, the AR-expressing PCa cell lines VCap and CWR-R1 were made enzalutamide resistant by long term cultures of at least six months or more that resulted in aberrant AR signaling of genomic and non-genomic pathways (Kregel et al., 2016). *LRP2* and MEGALIN expression also decreased in enzalutamide-resistant PCa cell lines VCap and CWR-R1, also indicating the loss of MEGALIN expression during disease progression to androgen-independence **(Figure 16A-B)**. These data identify putative interplay between the vitamin D and androgen axes alluding to dual hormonal regulation of *LRP2* expression, which establishes MEGALIN as a key player in a double feedback loop that functions to govern intracellular levels of 25D and T.

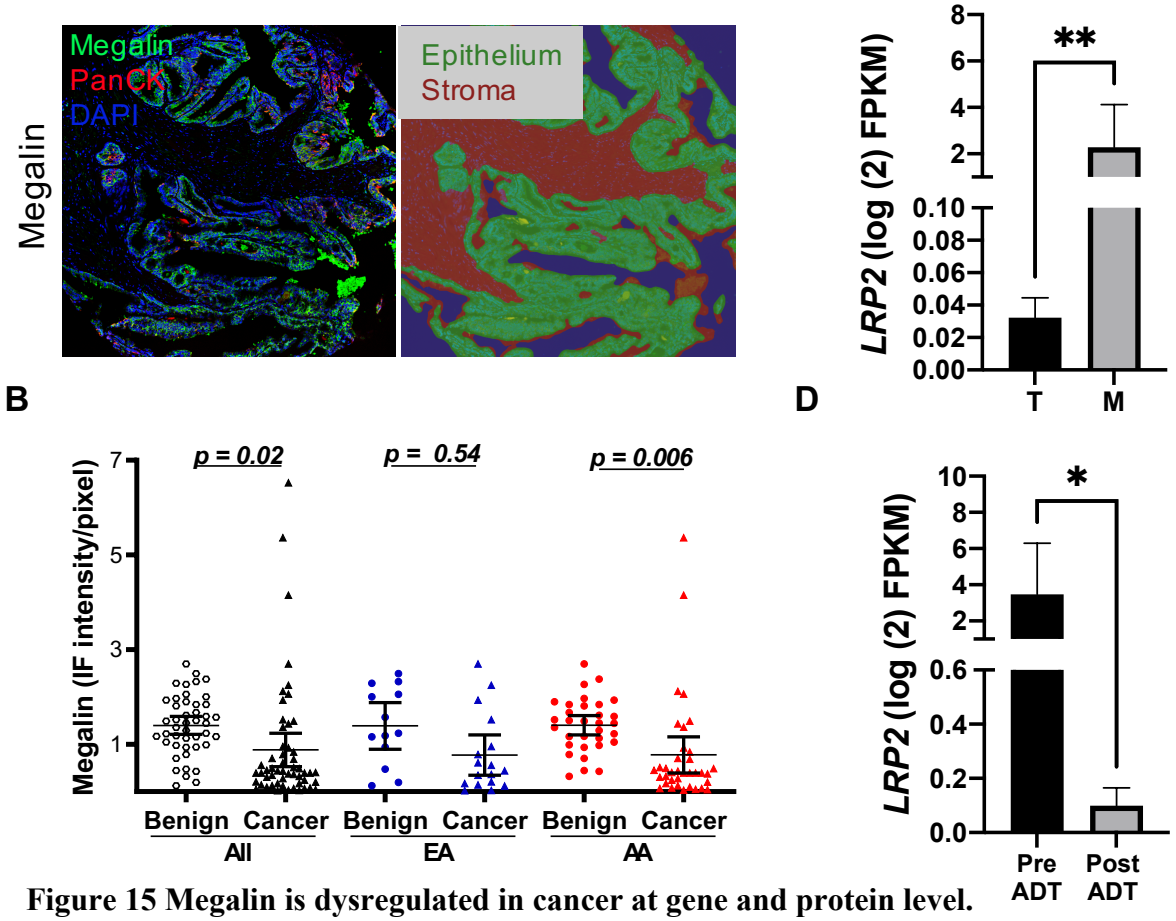

Figure 15 Megalin is dysregulated in cancer at gene and protein level.

A, digital quantitation of epithelial MEGALIN expression on a tissue microarray consisting of 118 prostate biopsy cores from 29 patients (EA, n = 9; AA, n = 20). Epithelial regions were segmented by PanCK staining and benign and cancer regions determined by a board-certified pathologist**. B,** immunofluorescence intensity per pixel for MEGALIN expresion. P values determined using unpaired t tests. **C**, gene expression of *LRP2* in primary tumors (n = 25) and metastases (n = 51) in an RNA-sequencing dataset. **D**, *LRP2* expression in metastases pre- (n = 33) and post- (n = 18). abiraterone/enzalutamide therapy (ADT) in an RNA-sequencing dataset (Bhanvadia et al., 2018; Pflueger et al., 2011; Robinson et al., 2015) . **C-D**, Graphs show mean log(2) FKPM (Fragments Per Kilobase of transcript per Million mapped reads) ± SEM of one experiment. P value determined using Wilcoxon signed rank test indicated with ***P < 0.0001.

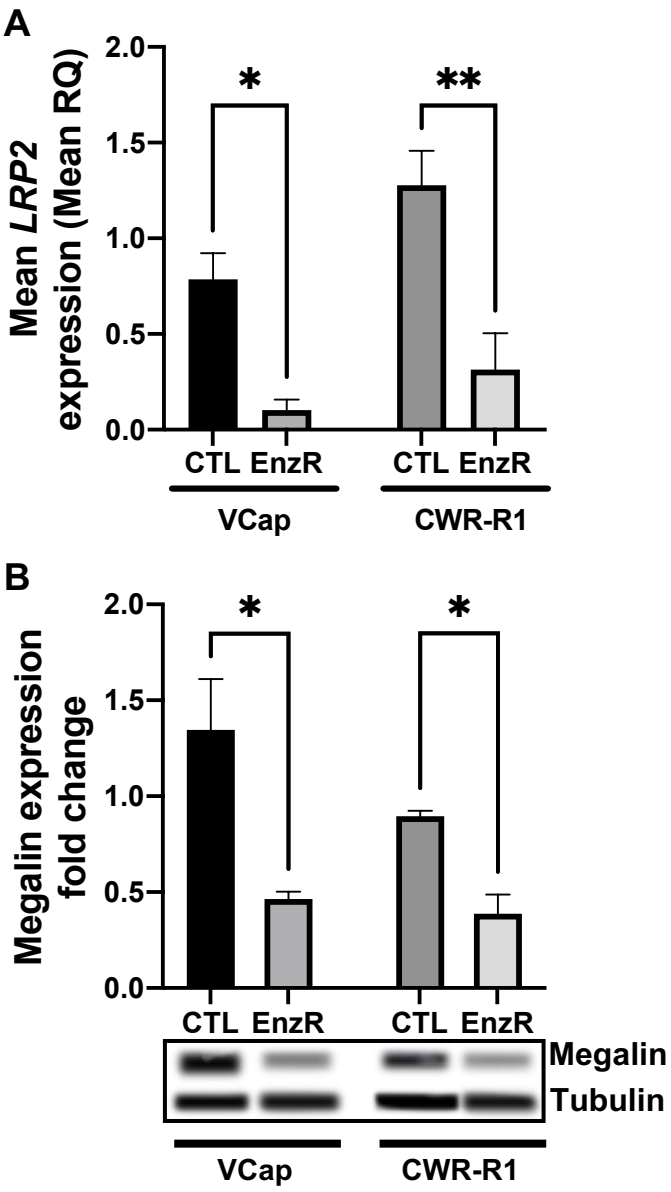

Figure 16 *LRP2* and Megalin expression in EnzR cells

A, RT-qPCR for *LRP2* expression in control and enzalutamide-resistant VCap and CWR-R1 cells. **B,** Megalin expression in control and enzalutamide-resistant VCap and CWR-R1 cells**.** Relative gene expression shown as relative quantity (RQ) normalized to *RPL13A.* P value determined using unpaired t test indicated with * $P < 0.05$; ** $P < 0.002$. Graphs represent mean ± SEM of two experiments with 2 replicates per experiment

CHAPTER III MATERIALS AND METHODS

A. Cell lines

LAPC4, CWR-R1, 22Rv1, and VCap were purchased from ATCC (VA, USA). Donald Vander Griend generously donated 957E-hTERT cells. VCap were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). LAPC4, CWR-R1, and 22Rv1cells were maintained in phenol-free Roswell Park Memorial Institute (RPMI) (Gibco) media supplemented with 10% FBS. LAPC4 and 22Rv1 cells were cultured in phenol-free RPMI (Gibco) supplemented with 5% charcoal stripped FBS (Millipore-Sigma) overnight, then serum starved for 1 hour prior to experimentation. 957E-hTERT cells were maintained and cultured in Keratinocyte Serum Free Media (KSFM). Primary prostate cells established from fresh male radical prostatectomy tissues were isolated as previously described (Giangreco et al., 2013; Nonn et al., 2006). Briefly, AA radical prostatectomy tissue from benign regions of the peripheral zone was collected according to UIC Internal Review Board-approved protocol and patients consented prior to surgery. Tissue histology was confirmed by a board-certified pathologist. Tissue was digested in collagenase/trypsin to single cells and cultured in Prostate Cell Growth Media (Lonza) to select for epithelial populations. When ~70% confluent, PrEs were cryopreserved singly and thawed prior to experimentation. All media was supplemented with 1% Antibiotic-Antimycotic (Thermo Fisher Scientific). All cells were cultured at standard 37°C with 5% CO2

B. Generation of enzalutamide-resistant cell lines

Greater than 10^7 CWR-R1, LAPC4, LNCaP and VCaP cells were plated and continuously cultured and maintained in 10 μM enzalutamide for at least 6 months prior towards any experimentation. During this time, > 90% of cells died (assessed visually), and resistant clones were pooled and maintained. Enzalutamide-resistant and matched parental cell lines were used within 10 passages of one another and maintained in culture for approximately the same amount

of time. Once resistant, cells were cultured in up to 20 μM enzalutamide without any change in phenotype.

C. Ex vivo prostate tissue slice culture

Prostate tissue (obtained in the same manner as described above) from a 5 mm dermal punch was sliced in to 300-micron sections using an Alabama Tissue Slicer (Alabama Research and Development), placed on aluminum alloy inserts within a 6 well plate and maintained in 2.5mL KSFM supplemented with 5% charcoal stripped FBS overnight until treatment. Slices were maintained and cultured overnight on a rotating thirty-degree angle at 37°C with 5% CO_2.

D. Gene expression by RT-qPCR

i. Vitamin D treatments. 2D culture: PrE's at 80% confluency were incubated with 10nM 25D (Enzo Life Sciences) for 16 hours. 3D culture: 3D culture: PrE organoids were seeded at 5000 cell per well in 33% matrigel (Corning) and cultured in KSFM supplemented with 5% charcoal stripped FBS for 7 days, then treated with 10nM, 50nM, and 100nM 25D in the presence and absence of 0.02ug/uL DBP for 24 hours. Prostate tissue slices were incubated with 50nM 25D in the presence and absence of 0.02ug/uL DBP (My Bio Source) in media supplemented with 5% charcoal stripped FBS for 16 hours.

ii. Testosterone treatments. For KLK3 detection, LAPC4 and 22Rv1 cells at 80% confluency were incubated with 50nM T with and without 250nM SHBG (Creative BioMart) and 1uM RAP (Enzo Life Sciences) for 16 hours. For *LRP2* detection, 22Rv1 cells at 80% confluency were incubated with 10nM T for 16 hours. Prostate tissue slices were incubated with 50nM T in the presence and absence of 250nM SHBG in media supplemented with 5% charcoal stripped FBS for 16 hours.

iii. RNA Isolation and RT-qPCR. RNA was isolated from cells and homogenized tissues with Trizol (Thermo Fisher Scientific) according to the manufacturer-supplied protocol. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Total RNA (500 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer-supplied protocol. Resulting cDNA was used for quantitative PCR amplification on a QuantStudio6 machine (Thermo Fisher Scientific) using gene-specific primers and FastStart Universal SYBR Green Master Mix (Millipore-Sigma). Reactions were run in duplicate and relative mRNA levels were normalized and calculated independently using the -ΔΔCt method (Livak and Schmittgen, 2001) to the expression of the housekeeping genes HPRT1. All primer sequences are listed below

GENE	FORWARD (5' -> 3')	REVERSE (5' -> 3')
LRP2	CTCCTGGGTGTGTGACCAA	CATCGGGGCAGTCTCTGAC
KLK3	TCCAATGACGTGTGTGCGCA	CCTTGATCCACTTCCGGTAA
CYP24A1	GGCAACAGTTCTGGGTGAAT	ATTTGAGGACAATCCAACA
CYP27B1	TTTGCATCTCTTCCCTTTGG	CTCAGGCTGCACCTCAAAAT
VDR	GACCTGTGGCAACCAAGACT	GAACTTGATGAGGGGCTCAA
SRD5A2	ATATATTGCGCCAGCTCAGG	GGAAATTGGCTCCAGAAACA
AR	TTGTGTCAAAAGCGAAATGG	CAATGGGCAAAACATGGTC
HPRT	TGCTGACCTGCTGGATTACA	CTGCATTGTTTTGCCAGTGT

Primer sequences

E. DBP-488 and SHBG-555 internalization

Alexa Fluor protein conjugation kits (Thermo Fisher Scientific) were used to conjugate 488 and 555 reactive dyes to both DBP and SHBG according to the manufacturer-supplied protocol, respectively. Aliquots of globulin conjugates were made and stored at -20C until use. PrEs, LAPC4 and 22Rv1cells were grown to 70% confluency in 8-well chamber slides. Cells were incubated with 0.05ug/uL DBP-488 with and without 10nM 25(OH)D or 0.2ug/uL SHBG-555 with and without 10nM T for 4 hours. Globulin conjugates and their respective hormones were pre-incubated in serum free media for thirty minutes to ensure formation of hormone-globulin complexes prior to treatments. Megalin function was inhibited using 1uM RAP. Cells were counterstained with Alexa Fluor 647 phalloidin and DAPI (Thermo Fisher Scientific). Sub-cellular internalization and localization of globulins was visualized under confocal microscopy

F. Dual-Luciferase Assays

957E-hTERT cells were grown to 70% confluency then co-transfected with 50ng *LRP2* promoter-driven Renilla luciferase reporter (Switchgear Genomics) and 1ng PGL4 Photinus pyralis (Promega) luciferase control reporter while simultaneously treated with 10nM 25D in the presence and absence of 0.02ug/µL DBP. LAPC4 and 22Rv1 cells were grown to 75% confluency then co-transfected with 250ng $(ARR)_2$ Photinus pyralis luciferase reporter (Zhang et al., 2000)and 100ng pRL Renilla luciferase control reporter (Promega) and treated with 50nM T in the presence and absence of 250nM SHBG and 1uM RAP. After 24 hours, Luciferase activity was measured using the Dual-Luciferase reporter assay system and the GLOMAX 20/20 (Promega) according to the manufacturer-supplied protocol.

G. Western blot analyses

957E-hTERT and 22Rv1 cells were grown to 60% confluency and treated with 50nM 1,25D and 50M R1881 (respectively) for 3 days before protein extraction. All cells were collected at 85% confluency using a cell scraper, lysed, then protein was isolated and quantified via Bradford assay. 25ug of protein was loaded into 4-12% gradient Bis-Tris protein gel 1.5mm (NuPAGE) and ran at 220V for 40 mins using 1X MOPS running buffer (NuPAGE). Protein was transferred to a PVDF membrane for 1 hour at 30V using 1X Transfer Buffer (NuPAGE) with 10% MeOH. Membrane was blocked for 1 hour using Odyssey Blocking Buffer in TBS (LiCOR) then probed with 1:1000 anti-MEGALIN rabbit monoclonal antibody (Boster Bio), and 1:1000 anti-tubulin monoclonal antibody (cell signaling) gently shaking overnight at 4C. Secondary antibody raised against rabbit (926-68071 LiCOR) was used at 1:20,000 for 1 hour at room temperature. All antibodies were diluted in Odyssey Blocking Buffer (TBS) + 0.2% tween 20. Blots were imaged using the Odyssey CLx imaging system (LiCOR)

H. Patient biospecimens

Fresh frozen prostate, whole blood, and serum were retrospectively collected from RP patients. Specimens from 60 patients were included for analysis: 30 from the UIC Hospital (Chicago, Illinois, USA) and 30 from the Cooperative Human Tissue Network Western Division at Vanderbilt University (Nashville, Tennessee, USA). Criteria for inclusion were self-declared race data, >500 mg of benign frozen prostatectomy specimen, serum, and whole blood. All patients had localized cancer without prior chemotherapy or hormonal therapy

J. LCM collection

Prostate specimens were removed from liquid nitrogen storage and thawed to –20°C in a cryostat prior to sectioning. RNase free conditions for LCM was carried out as previously described by our

group (38). Briefly, 3 or more 10-micron cryosections per specimen were placed onto PEN membrane 4.0 μm LCM slides from Leica Biosystems and stained with Toluidine blue in RNA and RNase free solutions for visualization under the microscope. An adjacent section was H&E stained for markup by a board-certified pathologist. Only benign epithelium >1 cm from cancer was used for this study. LCM was done for 1 hour only to prevent RNA degradation and resulted in 100–200 acini per specimen. Tissue was stored in lysis buffer (RNAqueous-Micro kit, Ambion) at –80°C until ready for RNA isolation.

i. RNA extraction and amplification

Extraction and isolation of RNA from the LCM samples was completed using the RNAqueous-Micro kit, and the RNAqueous-Micro procedure for LCM protocol was followed with DNase treatment. Quantity and quality of RNA was assessed via NanoDrop ND-1000 (Thermo Scientific), and yields ranged from 99.6 ng/μl to 602.8 ng/μl. For each sample, 10 ng of RNA was used for whole transcriptome amplification using the Ovation PicoSL WTA System V2 (NuGEN) and purified with the DNA Clean & Concentrator-25 kit (Zymo Research). Quantity and purity of cDNA was also assessed via NanoDrop.

ii. Gene expression by DNA microarray

Gene expression analysis Amplified cDNA from 26 LCM patient samples (13 EA,13 AA) was biotin labeled using the Encore Biotin Module (NuGEN) and hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix). Use of microarray enabled optimal and accurate quantification of gene expression, given the limited nature of the LCM samples. Hybridization and chip scanning were completed by The Core Genomics Facility at the UIC using standard Affymetrix protocols. The samples were divided into two batches for labeling, hybridization, and scanning, and samples were evenly distributed across batches

by race and collection site to mitigate any potential bias. Principal component analysis confirmed no batch effect was present. Microarray data from CEL files were read, background corrected, and normalized. Robust multi-array averages were generated using the Affy (58) and Oligo (59) packages from Bioconductor (https://www.bioconductor.org/) in R (https://cran.r-project.org/). The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (60) and are accessible through GEO Series accession number GSE91037.insight.jci.org

I. T and DHT Measurement

i. Calibration curve - Standard compounds T and DHT (1 mg/ml in MeOH), internal standard (IS) d3T (0.1 mg/ml in ACN) were purchased from Cerilliant (Round Rock, TX, USA). Spiking solutions of T and DHT used to prepare calibrators were diluted with MeOH to 10 µg/ml, 5 µg/ml, 1 µg/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, and 6.25 ng/ml. The working solution of IS was prepared by diluting the original stock to a final concentration of 500 ng/ml with ACN. Nine calibrators: 0.0625, 0.125, 0.25, 0.5, 1, 5, 10, 50 and 100 ng/ml in MeOH were used to establish calibration curves. The final concentration of IS was 50 ng/ml. All solutions were stored at -40°C in amber vials. Curves were fitted by linear regression with a weighting factor of 1/x.

ii. Tissue homogenization and extraction - Approximately 20 to 200mg of tissue from each patient was bead homogenized using the Mikro-Dismembrator II (Handelskontor Freitag, Germany), collected into pre-weighed tubes chilled on dry ice, and resuspended in 5 volumes of 10% MeOH (m : v) immediately prior to extraction. Sample homogenate (500 µl) was transferred into glass tube, 10 µl of IS working solution was immediately spiked in. After mixing each sample was extracted 3 times, each time using 3 volumes of hexane : ethyl acetate (60 : 40, v:v) followed by vigorous vortexing (10 sec x 3). After

vortexing samples were centrifuged at 2000 pm for 5 min, the organic layer from each extraction was collected, combined and brought to dryness under high purity nitrogen flow. The residue was reconstituted into 1 ml of MeOH : H2O (20 : 80, v:v) and subjected to solid phase extraction (SPE) using ISOLUTE C18 SPE cartridge (100mg, 1 ml) following vendor's protocol. Briefly, prior to sample loading, cartridges were activated with 3 ml of MeOH, and equilibrated with 3 ml of H2O. Once samples were loaded onto the cartridges 2 ml of H2O was used to wash. The target analytes were then eluted into 2 ml of MeOH. This elute was dried under nitrogen then reconstituted into 100 µl of MeOH for LC/MS analysis.

iii. Serum extraction - 100 µl of human serum was used for extraction. For samples less than 100 µl, the volume of the sample was measured, and the entire sample was taken for the measurement. After spiking in 10 µl of IS working solution, serum samples were extracted and reconstituted following the same procedure described above. Samples started with less than 100 µl were reconstituted in 50 µl instead of 100 µl of MeOH.

J. LC-MS/MS analysis - Quantification of T and DHT was achieved using SCIEX Qtrap 6500 spectrometer coupled with Agilent 1290 UPLC system. Five µl of sample was injected and eluted by Waters ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) maintained at 45 degrees, at a flow rate of 450 µl/min. Elution started with 60% of mobile phase A (5% MeOH in H2O, 0.1% FA), followed by a linear gradient increase of mobile phase B (ACN with 0.1%FA) from 40 to 80%. MS data were acquired by MRM scan at positive mode. The ESI spray voltage and source temperature was set at 5.0kV and 450 degrees. T, DHT and D3T were detected by monitoring their transitions to signature product ions 289>97 (T), 291>255 (DHT), and 292>97 (D3T) respectively. Data were analyzed using Analyst software.

K. Vitamin D Metabolite Measurement

i. Extraction - Samples were weighed and spiked with the internal standards d6-1,25(OH)2D3, d3-25(OH)D2 and d3-25(OH)D3, then saponified with methonalic potassium hydroxide. Both deuterated and nondeuterated neat reagents were purchased from Sigma-Aldrich. After saponification for 2 hours, the samples were vortexed and extracted with methylene chloride. The organic layer was dried and reconstituted with hexane/methylene chloride, and both 25(OH)D and 1,25(OH)2D were isolated on a 0.5 g silica SPE column. The metabolites 1,25(OH)2D and 25(OH)D were eluted and then reconstituted into vitamin D–stripped serum (DiaSorin).

ii. 25D - From this point, the 25D was extracted via liquid-liquid extraction using hexanes along-side a serum-based standard curve with 972a serum (National Institute of Standards and Technology) serum 25D controls. The samples were reconstituted into LCMS-grade methanol and LCMS-high purity water both with 0.1% formic acid, and then injected onto an Agilent 1290 HPLC with an Agilent C18 Poroshell Column coupled to an Agilent 6460 Triple-quad mass spec with electrospray ionization source (ESI) in positive mode and analyzed using Masshunter software. The 25(OH)D measurements had a percent coefficient of variation of 8.6% for tissue and 6% for serum.

iii. 1,25D - The samples, standard curve, and 1,25(OH)2 D QC calibrator (DiaSorin) were then extracted from the serum and serum reconstitution by acetonitrile protein precipitation, followed by SPE isolation using C-18OH DiaSorin columns. Samples were eluted and dried in a savant dryer. The 1,25(OH)2D fraction from the samples/standard curve/QC were derivatized using 100 µl of 0.75 mg/ml PTAD in acetonitrile for 2 hours at room temperature. The samples were quenched with 50 µl LCMS-grade water, loaded into

LCMS vials, and injected onto an Agilent 1290 HPLC with an Agilent ZORBAX C18 Eclipse plus RRHD column coupled to an Agilent 6460 Triple-quad mass spec with ESI in positive mode and analyzed using Masshunter software. The 1,25(OH)2D assay had a percent coefficient of variation of 13.0% for tissue and 10.8% for serum.DBP

L. Tissue microarray immunostaining and analysis

A formalin fixed, and paraffin embedded human tissue microarray containing 118 prostate biopsy cores from 29 patients (20 AA, 9 EA) and consisted of at least 2 benign and cancer cores from each patient. Pathology of and mark-up of each core was completed by a board-certified pathologist. Sections of 5 microns were incubated with rabbit polyclonal anti-*Lrp2*/MEGALIN antibody (ab76969) diluted 1:100 or rabbit polyclonal anti-VDR antibody (C-20) diluted 1:100 (abcam, Cambridge, UK), and mouse monoclonal anti-PanCK (AE1/AE3) diluted 1:2000 then incubated with secondary antibody AlexaFluor-488 goat anti-rabbit diluted 1:200 and AlexaFluor-555 goat anti-mouse diluted 1:200 (Life Technologies, Carlsbad, CA, USA), counterstained with DAPI and imaged on the PerkinElmer Vectra3. Epithelial areas were identified and segmented by PanCK marker automatically using PerkinElmer Inform software (version 2.3) and adjusted manually to ensure accuracy. Megalin and VDR fluorescence intensity were quantified and reported as average intensity per pixel of the segmented area of each core using Inform software.

M. RNA-Sequencing datasets

We obtained raw RNA-Seq FASTQ files of 25 prostate tumors, 3 benign glands, and 51 annotated metastases via dbGAP (phs000310.v1.p1 for tumors and benign glands; phs000915.v1.p1 for metastases; (Pflueger et al., 2011; Robinson et al., 2015). These datasets were chosen based upon the quality of mRNA, sequencing depth, and annotation. The tumor datasets are from patients who had undergone prostate surgery without prior therapy, and the metastases are from patients with

castration-resistant prostate cancer, some of which had received taxane, enzalutamide, or abiraterone treatment (Pflueger et al., 2011; Robinson et al., 2015). The quality of raw reads was accessed by FastQC (v0.11.4). All reads were mapped to the human genome assembly (NCBI build 19) using STAR (v2.5.1b). Alignment metrics were collected by Picard tools (v2.8.1) and RSeQC (v2.6.4). Transcripts were assembled from the aligned reads using Cufflinks and combined with known gene annotation. The expression level of transcripts was quantified using FPKM (Fragments Per Kilobase of transcript per Million mappedreads)-based and read count–based methods.

CHAPTER IV: DISCUSSION

This study follows up on our recent finding that MEGALIN protein is expressed in the membrane of prostate epithelium (Richards et al., 2017). It is well-established that MEGALIN mediates endocytosis of DBP-bound 25D in the kidney (Christensen and Birn, 2002; Christensen et al., 2012; De et al., 2014; Moestrup and Verroust, 2002). To determine if this process extends to the prostate, we used immortalized cells, PrEs, and prostate tissue explants for mechanistic examination of MEGALIN-mediated DBP-25D and SHBG-bound T uptake and determined how this mechanism is regulated in response to these steroid hormones

In our previous study, we suggested that MEGALIN is part of a compensation pathway to increase intra-prostatic vitD metabolites when the patients are deficient in vitD, based on our observation that prostatic expression of *LRP2* negatively correlated with serum 25D (Richards et al., 2017). The *in vitro* and *ex vivo* experiments confirmed that 25D decreased *LRP2* gene expression in PrEs and tissue explants. *LRP2* promoter activity was also inhibited by 25D. Our findings complement those of Chapron et al who also recently suggested compensation in the kidney to upregulate MEGALIN in response to low renal vitD (Chapron et al., 2018). Additionally, skeletal muscle cells *in vitro* pretreated with 1,25D had significantly decreased 25D uptake (Abboud et al., 2018), also supporting downregulation of MEGALIN by high levels of vitD metabolites. These data show that vitD metabolite concentrations regulate *LRP2* expression in multiple tissue types. Taken together, this builds evidence for active extra-renal MEGALIN and a regulation of tissue levels of hormones via import of globulin-bound vitD metabolites rather than reliance on passive diffusion of free hormone.

The activity of MEGALIN was confirmed by internalization of DBP-bound 25D in prostate epithelium. In our study, 25D bound to DBP induced expression of CYP24A1 and decreased *LRP2* expression in PrE organoids and prostate tissue slices demonstrating that MEGALIN may be functional and responsible for internalization of DBP-bound 25D in the prostate. VDR translocated to the nucleus after treatment with DBP-25D in 957E-hTERT cells, indicating bioactivation of

imported 25D. Additionally, PrEs internalized DBP-488 with 25D and this uptake was diminished after coincubation with RAP. These findings show that MEGALIN may be capable of endocytosing DBP-bound 25D in prostate epithelium. Although these data indicate DBP-bound 25D uptake, the ratio of DBP to 25 used was not physiologically relevant, so much of the CYP24A1 induction observed would be induced by passively diffused 25D.

Megalin internalizes many ligands, but the most prostate-relevant is SHBG, which has been demonstrated in rat yolk sac (Hammes et al., 2005), and fetal hippocampus (Caldwell et al., 2007). Megalin is also expressed in rat epididymis, uterus, and placenta (Zheng et al., 1994) and the *Lrp2* knock-out is lethal for 95% of the mice and the few surviving mice display impaired descent of the testes into the scrotum and other defects consistent with sex steroid disruption, findings which support a role for MEGALIN regulating T in steroid-responsive tissues (Caldwell et al., 2007; Hammes et al., 2005; Nykjaer et al., 1999). We showed that LAPC4 and 22Rv1 cells internalized SHBG-555 + T that was blocked by RAP, endocytosed biologically active T that induced KLK3 expression, and blocked this activity with RAP by inhibiting the translocation of AR to the nucleus. In these same cells transfected with an $(ARR)_2$ luciferase reporter treated with SHBG-bound T resulted in >1000-fold induction of promoter activity. Prostate tissue slice explants treated with SHBG-T also induced expression of KLK3, confirming the ability for prostate to utilize SHBG-bound T in the most current physiologically relevant model. These new observations are not only paramount to fully understanding androgen access and its influence on normal and disease prostate biology, but how this mechanism is regulated is of the utmost importance. Using a similar experimental approach, we also demonstrated that *LRP2* and MEGALIN expression are inhibited by androgens in the same models mentioned above. Additionally, this mechanism was irrefutably validated *in vivo* as *Lrp2* increased in luminal cell populations of castrated mice and then decreased upon androgen restoration from an RNA-seq data set.

In a case control study following our observations of MEGALIN expression in patients with low to moderate Gleason scores treated with primary androgen deprivation therapies, Holt et

al analyzed genetic polymorphisms within *LRP2* to determine if MEGALIN could alter uptake of sex steroid hormones and how it associates with PCa risk and prognosis. Forty-haplotyped single nucleotide polymorphisms (htSNP) were analyzed in 553 Caucasian men diagnosed with PCa between 65 and 40 year of age and 543 controls. The results showed an evidence of PCa development between the forty haplotypes. There was, however three htSNPs significantly correlated with both recurrence/progression and lethality of PCa. Recurrence and risk alone associate with five more htSNPs, and six additional htSNPs were implicated as modifiers by primary ADT. Two more htSNPs significantly correlated with altered risk of PCa lethality. These preliminary results infer that *LRP2* variants may modify risk of lethal PCa and progression/recurrence that may be race specific.

The significance of these discoveries is momentous and will pervade our learning of human anatomical hormonal landscapes. PCa is a disease that correlates with age, and in all aging men, T levels decline and SHBG levels increase (Feldman et al., 2002; Gann et al., 1996a) as does adipose tissue especially in low and middle-income populations (Mancuso and Bouchard, 2019). T is converted to estradiol (E2) by the enzyme aromatase that is expressed in adipose tissues where most aromatization occurs (Huhtaniemi, 2018). E2, another carcinogenic steroid hormone binds SHBG and is known to interact with T to cause PCa in animal models (Bosland, 2013; Hayward et al., 2001; Ricke et al., 2006). In parallel, the ratio of E2/T increases as does the prevalence of PCa in AA men only (Vermeulen et al., 2002). And as we continue aging, vitD deficiency also progresses as 1,25D production decreases by 50% resulting from reduced renal 1-apha-hydroxylation of 25D (Kinyamu et al., 1996) along with a two-fold decrease of D3 synthesis resulting from age-related decline of cutaneous 7DHC expression (MacLaughlin and Holick, 1985). It is challenging to predict how these fluctuations would exert their transcriptional regulations on VDRE, ERE, and ARE half sites within the *LRP2* promoter **(Figure 9)** and it is unknown how this all will coalesce as MEGALIN levels and functionality may change in aging

males of differing races, and whether the receptor can somehow be targeted for therapy if necessary and/or possible.

Our prior study showed that prostate *LRP2* expression positively correlated with %WAA of the patients (Richards et al., 2017). As 82% of all African Americans in the United States are deficient in vitD (Forrest and Stuhldreher, 2011), this suggests that expression of *LRP2* will accordingly be higher, leading to more import of SHBG-bound T and E2 in that population. In our cohort we observed that AA men indeed did have higher levels of DHT in prostate tissue compared to EA men. In the serum, T was the dominant metabolite with very low DHT, whereas prostatic T levels were undetectable in most men, consistent with uptake of T into the cells followed by rapid conversion to DHT by 5α-reductase. We also observed lower levels of serum T in the AA men compared to EA men of our cohort which has been associated with aggressive PCa in late-stage disease (Schatzl et al., 2001) and further supports active transport of SHBG-T in the prostate rather than passive diffusion of T. This finding also provides additional insight regarding progression to CRPC. Our tissue findings differ with prior studies that found no racial discrepancies in prostatic T (Mohler et al., 2004a) and significantly higher levels of free circulating T or DHT, but higher SHBG and androstenedione in prostate of black men compared to white men (Richard et al., 2014). The reasons for this are not apparent and may be due to differential methods (ELISA versus LC-MS/MS) and sample preservation. However, our finding of elevated prostatic DHT in the AA men may directly contribute to increased incidence of early-onset PCa and PCa mortality among AA men.

Tying this back to vitamin D status, we observed that serum 25D and prostatic DHT were significantly inversely correlated. This relationship in combination with the findings of our prior study, and our current finding of androgen inhibiting *LRP2* expression, outlines an intricate yet detrimental interaction between the androgen and vitD axes that characterizes the adverse effects of vitD deficiency and increased MEGALIN-mediated import of androgen in men of West African descent. Although our results were significant, we acknowledge there is the limitation

of underrepresentation of vitD-replete AA patients and vitamin D-deficient EA patients in the cohort, which is required to discern ancestry from deficiency.

Given the dependence of PCa on androgens, we examined MEGALIN protein and *LRP2* gene expression in multiple cohorts of PCa patients on a tissue microarray **(Figure 15A).** Megalin protein was markedly lower in cancer areas compared to benign in radical prostatectomy samples **(Figure 15B),** an observation not previously made. The decrease in MEGALIN in early cancer may limit androgen uptake and therefore prime a maneuver towards an androgen-independent state. Although MEGALIN was decreased in localized disease, *LRP2* expression was increased in metastases **(Figure 15C)**. In CRPC following treatment with androgen ablation, we observed that *LRP2* expression was virtually absent **(Figure 15D),** which is consistent with the hormone independence of this stage of the disease. And this observation was replicated *in vitro* using enzalutamide-resistant VCap and CWR-R1 cells. We measured *LRP2* and MEGALIN expression in both cell lines and found that both mRNA and protein were absent compared to their parental cell lines **(Figure 16A-B)** which is again consistent with the androgen independent state of CRPC. Altogether, these data may characterize a dual role for MEGALIN. If MEGALIN expression is a phenotype of differentiated epithelium, then the decrease in localized disease may be a result of the de-differentiation of the cancer.

CHAPTER V: CONCLUSIONS

It is well established that all steroid hormones follow the principals of the FHH **(Figure3).** This endocrinological doctrine established by Carl Mendel in 1989 states that the activity of a hormone is based on its bioavailable fractions, while bound fractions (25D ~88%, T~ 70%, E2~23%) are not accessible to tissues. (Mendel, 1989b). The FHH is commonly accepted and is believed that free levels of systemic hormones should reflect concentrations present in tissues. And it is well-known that as men age, T levels decline, SHBG levels increase and so does the ratio of bound/free T (Vermeulen et al., 2002). However, in the present study we found that serum and prostate tissue androgen metabolites from AA and EA did not mirror; an observation that is not the first of its kind

Cook et al questioned this theory and tested if systemic and prostatic sex steroid hormone levels (including T and DHT) are concordant in a cohort of 251 PCa cases. The main conclusions of the study were that circulating T and DHT concentrations did not correlate with intraprostatic concentrations, suggesting that systemic levels are poor measures of the intraprostatic landscape (Cook et al., 2017). The significance of unequal serum and prostate androgen levels observed in this study also opposes the FHH and suggests MEGALIN as an additional entry mechanism for systemic sex steroids in reproductive organs as was demonstrated in MEGALIN-deficient mice (Hammes et al., 2005). This clinical observation by Cook et al. should serve as the impetus to begin minimizing the dearth of understanding of circulating androgens and risk of PCa development

Also in this present study, we identified MEGALIN as an entry mechanism for SHBG-bound T into the prostate and discovered that *LRP2* expression is inhibited by androgens. Androgen-mediated inhibition of *LRP2* gene expression was clinically inferred in past studies that determined low serum T associates with PCa risk or Gleason grade (Schatzl et al., 2001; Stattin et al., 2004). In a related prospective analysis of 279 patients referred for first or second biopsy on suspicion of PCa from digital rectal exam or elevated PSA, free and bioavailable T, and SHBG,

were all recorded to determine a cut-off point of hormone concentrations to assess risk of PCa. 84 of the 279 men who received abnormal DRE, had high SHBG levels > 66.25nM and low bioavailable T < 104 ng/dl associated with adenocarcinoma of the prostate. The authors concluded that high SHBG and low bioavailable T were linked to a 4.9-to-3.2-fold risk of detection of disease on prostate biopsy. (Garcia-Cruz et al., 2013). In this setting, abated AR inhibition of *LRP2* could upregulate MEGALIN expression in an environment primed for expeditious SHBG-bound T internalization. This would disprove the FHH and provide existing adenocarcinomas a proliferative advantage to advance to more aggressive stages. (Garcia-Cruz et al., 2013).

However, in favor of the FHH, many studies have established a protective role for SHBG against breast cancer by regulating cellular entry of bioavailable E2, which is known to promote proliferative and anti-apoptotic effects in the breast (Fortunati et al., 2010). In a meta-analysis of nine prospective studies of postmenopausal women including 1765 healthy women and 663 who developed breast cancer, SHBG alone inversely associated with risk of breast cancer. (Key et al., 2002). In a more recent case-controlled study, SHBG levels were significantly lower in postmenopausal women with breast cancer compared to controls, whereas in premenopausal women who developed breast cancer, no significant difference in SHBG was observed. (Kaaks et al., 2005) In relation, SHBG levels were shown to decrease from pre-menopausal to post-menopausal women in good health. Therefore, SHBG references ranges have potential to predict individuals at high-risk for breast cancer development and aid direction of treatment choices in post-menopausal women (Park et al., 2020). Unfortunately, studies investigating associations with SHBG and breast cancer during pregnancy are scare, but given the increase of SHBG and T during gestation, it is possible that SHBG regulates the bioavailable fractions of both E2 and T in a protective manner

In vitro analyses of SHBG and E2 interactions echo the chemo-preventive role against breast cancer cells. In MCF7 cells, only unliganded SHBG is capable of binding to cell surface, which then allows E2 to bind SHBG (Khan et al., 1990) Unliganded SHBG binding to G-protein

membrane receptors triggers intracellular signaling cascades mediated by cAMP that target PKA which inhibit MAP kinase-induced cell proliferation. (Fortunati et al., 1996). Liganded SHBG was also proven to abrogate E2-mediated erk-induced anti-apoptotic activity in MCF7 cells. (Catalano et al., 2005) The abrogation of E2-mediated carcinogenic effects is inhibited by SHBG-mediated downregulation of ERα, a result that may induce apoptosis and antiproliferation. (Truchet et al., 2000). Collectively, extracellularly bound SHBG inhibits E2-mediated effects *in vitro*.

Circling back to vitD and PCa, risk of lethal PCa is inversely associated with higher total (bound and unbound) serum 25D levels (Shui et al., 2012). In a follow-up prospective case control study from the same group consisting of 156 matched PCa and controls, bioavailable 25D and DBP-bound 25D levels were compared to determine the risk of advanced and lethal PCa based on interactions of bioavailable 25D and DBP. The authors found that bioavailable 25D inversely associated with risk of advanced and lethal PCa to a lesser extent compared to total 25D. (Yuan et al., 2019). Given our proven MEGALIN-mediated DBP-bound 25D import in physiologically relevant explant models, these findings are discordant with the FHH, and they infer DBP to be a protective modifier against aggressive and lethal PCa by further exerting the chemopreventive properties of vitD by way of receptor-mediated endocytosis and bioactivation of 25D in prostate cells.

AA men express the GC1F DBP variant in a homozygote fashion in a high frequency, while other variants and heterozygote genotypes are more frequent in whites. The GC1F variant, when homozygous, has the highest affinity to 25D compared to other DBP variants, but also associates with the lowest amount of DBP in humans compared to other variants (Chun et al., 2014; Powe et al., 2013) . In another prospective study including 452 controls matched to 226 PCa cases that analyzed the links between 25D, DBP, and the DBP-25 molar ratio with PCa risk in AA men, researchers found that serum 25D did not corelate with any overall prostate cancer risk. Interestingly, higher rather than lower systemic DBP inversely correlated with PCa risk in AA men independent of 25D status (Layne et al., 2017). As previously stated, MEGALIN internalizes DBP-

bound 25D in the prostate, and AA men have a higher frequency of the GC1F DBP variant that has a higher affinity to 25D compared to other DBP variants (Chun et al., 2014; Powe et al., 2013). Uptake of bound-25D in this setting may result in accelerated uptake of 25D, leading to inverse associations of 25D with the risk of PCa observed in this cohort of AA men.

The interplay between vitD status, MEGALIN and cancer may extend to breast cancer another hormone-sensitive disease responsive to vitD chemoprevention. This was shown in a randomized control trial that proved vitD supplementation to participants decreased breast cancer incidence and progression, an effect with the greatest reduction in women with healthy body composition (Chandler et al., 2020). *In vitro* evidence against the FHH demonstrated in various extra-renal vitD-sensitive organ systems have showed that HME immortalized breast epithelial cells can internalize DBP-bound 25D to induce a CYP24A1 reporter and demonstrated that T47D breast cancer cells treated with 25D in the presence of 10% FBS, a rich source of DBP, displayed induction of CYP24A1 expression (Rowling et al., 2006). Using confocal microscopy, the same group demonstrated that internalization of an Alexa-conjugated DBP could be visualized in HME and T47D breast cancer cells (Chlon et al., 2008).

In other cell types, Gao et al also showed that MEGALIN is required for induction of CYP24A1 mRNA by 25D-DBP import in human mesenchymal stem cells achieved using siRNA-mediated knockdown of *LRP2*, (Gao et al., 2019). Furthermore, suggestive MEGALIN-mediated 25D uptake occurred in the fetal hippocampus of rats (Caldwell et al., 2007). The sum of these findings refutes the notion of passion diffusion as the sole cellular entryway of 25D.

Although there remains no definitive evidence of racial differences in serum steroid hormones, one study over an 8-year period found that AA men aged 24-41 had higher serum T concentrations compared to age-matched EA men, but this difference was no longer apparent after adjustment for age and waist circumference. (Gapstur et al., 2002). An earlier study found a 15% increase in AA serum T levels (Ross et al., 1986). However, more recent studies with modern methods have not demonstrated any differences in systemic T. Another study found that non-

Hispanic AA pre-pubertal boys and men have higher levels of serum E2 and lower levels of SHBG compared to Mexican American boys and men and non-Hispanic whites (Abdelrahaman et al., 2005; Rohrmann et al., 2007) Although these levels fluctuate with age, they may contribute to earlier onset of PCa in AA.

To this date, there have been three studies that measured and compared AAs and EAs androgen metabolites, two of which reported no differences in prostatic T or DHT although the Mohler study reported higher SHBG in AAs (Marks et al., 2006; Mohler et al., 2004b). The third study is the present, where we report that AAs had lower levels of T in serum and higher levels of DHT in prostate tissue compared to EA men – a finding first of its kind.

The significance of these observations is disconcerting and should be considered carefully. It is generally believed that T alone can promote PCa progression based on the Huggins study (Huggins and Hodges, 1941) but the etiology of the disease in not entirely understood. The long-standing belief is that the prostate is dependent on androgens for development and growth and PCa is an androgen-dependent disease. And so, it is believed that androgens stimulate cell proliferation for a suitable duration for oncogenic alterations to occur. But there is no direct evidence for this. In surgically castrated rats, devoid of spontaneous adenocarcinomas, the prostate involutes and secretory function ceases until androgens are restored, which returns the prostate back to its normal proliferative function within 4 days. There are no human data on the influence of androgen treatment on prostate cell proliferation, there are only reports of serum levels of PSA resulting from androgen administration, which do not accurately depict cell proliferation. (Bosland, 2013). Overall, our understanding of androgens and PCa is limited only to androgenic progression of the disease. There exists no definitive clinical evidence that systemic androgen levels causatively associate with development of PCa

An existing theory of PCa etiology also derives from the chemo-promoting cooperative effects of E2 and T, which mostly were conducted in animal models (Bosland, 2013). E2 is known to induce cell proliferation and cause inhibition of apoptosis (Fortunati et al., 2010), but E2 can be

converted to catecholestrogens that quickly form intensely reactive estrogen quinones and semiquinones by redox recycling. These reactive metabolites redox-recycle themselves and adduct DNA creating reactive oxygen species (ROS) that cause lipid peroxidation that form lipid hydroxides, both of which damage and mutate DNA (Cavalieri et al., 2000). Other estrogen metabolites like 4-hydoxyestradiol adduct DNA quickly forming apurinic regions of DNA prone to mutations derived from error-prone DNA-repair machinery (Mailander et al., 2006). Collectively, these observations and data infer that for androgens to promote PCa, T aromatization must occur so that both T and E2 can cooperate to develop adenocarcinoma of the prostate. Since this theory was only proven in animals, translation to human therapies is warranted however this would require multidisciplinary clinical trial testing, which is not so easily achievable.

In conclusion, our *in vitro* and *ex vivo* data show that MEGALIN is functional in the prostate and responsible for transporting T bound to SHBG and 25D-bound to DBP into the cell. We also show that MEGALIN expression is negatively regulated by vitD and androgen, and in times of deficiency, is upregulated, potentially increasing import of 25D, T, and E2. If this is true, it may signify a once evolutionary protective compensatory mechanism of vitD gone awry, increasing the likelihood of androgen and estrogen import therefore increasing risk of carcinogenic events contributing to disparities in PCa aggressiveness (and perhaps PCa development) that plagues AA men. This study further contributes to our understanding of extra-renal activity of MEGALIN in the prostate and relates to the disparity of PCa in AA men as we found MEGALIN to be negatively regulated by vitD metabolites and AA men are prone to vitD deficiency. Our observation that intraprostatic DHT is higher in AA men that negatively correlates with vitD status supports a chemopreventive role for vitamin D status by its regulation of MEGALIN. Finally, our current study complicates the FHH and confirms that globulin bound hormones are accessible to the prostate by MEGALIN-mediated endocytosis – results that will redefine treatment modalities for hormonally-related development carcinogenesis and progression.

CHAPTER VI: FUTURE DIRECTIONS

The regulation of *LRP2*/MEGALIN expression and functionality warrants further exploration. This could be achieved using a prostate specific *LRP2* knock out mouse that utilizes Cre-Lox recombination, with a Cre recombinase under a Probasin promoter. Firstly, deletion of the *LRP2* gene should be investigated to determine if MEGALIN imports 25D, T, and E2. This should first be performed by knocking out *LRP2* as early in the mouse's life as possible, then again in a mature mouse. Secondly, and if MEGALIN has been shown to import 25D, T, and E2, a vitD deficient diet should be used to determine if vitD regulates *LRP2*/MEGALIN expression. Multiple endpoints should be explored for both questions including histological observations using H&E stains, gene and protein expression using RT-qPCR and immunohistochemistry, and ultra-high performance liquid chromatography tandem mass spectrometry of 25D, T , and E2, all of which performed using mouse prostate material after deletion of *LRP2* and after the mice have been on a vitD-deficient diet for at least four months. These techniques would provide a comprehensive approach to determine if MEGALIN imports hormones and if *LRP2* is regulated by vitD and how this regulation affects vitD, T, and E2 import in mouse prostate.

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APPENDIX

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

February 24, 2021

Larisa Nonn
Pathology
M/C 847

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Nonn:

The protocol indicated below has been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago on 2/11/2021. *The protocol was not initiated until final clarifications were reviewed and approved on* ***02/24/21.*** ***Protocol expires 3 years from the date of review 02/11/24.*** *This protocol replaces protocol 2018-018, which has been terminated.*

Title of Application: **Lentriviral Transduction of cDNA and shRNAs into Cell Lines**

IBC Number: **21-005**

Highest Biosafety Level: 2

You may forward this letter of acceptable IBC verification of your research protocol to the funding agency considering this proposal. **Please be advised that investigators must report significant changes in their research protocol to the IBC office via a letter addressed to the IBC chair prior to initiation of the change. If a protocol changes in such a manner as to require IBC approval, the change may not be initiated without IBC approval being granted.**

Thank you for complying with the UIC's Policies and Procedures.

Sincerely,

Alan McLachlan, PhD
Chair, Institutional Biosafety Committee
AM/*vp*
Cc: IBC file

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VITA

Jason Garcia

EDUCATION

University of Illinois Chicago, Chicago IL
PhD, Pathology, 2021

Northeastern Illinois University, Chicago IL
Bachelor of Science (B.S.) 2016

AWARDS AND HONORS

Chancellor's Student Service and Leadership Award (2020): for recognition as an outstanding student leader and student volunteer

American Association for Cancer Research (AACR) Minority Scholar in Cancer Research Award (2019): awarded $1500 for increasing the visibility and recognition of minority scientists involved in cancer research

Society for Basic Urologic Research (SBUR) travel award (2018 & 2020): awarded $500 for outstanding submitted abstract

Vitamin D Workshop Travel Award (2019): awarded $500 on the basis of scientific merit for a submitted abstract

PUBLICATIONS

Garcia J., (In preparation) "Vitamin D deficiency increases Megalin and associates with higher intra-prostatic androgens in African American men"

McCray T., Pacheco J.V., Loitz C.C., Garcia J., Baumann B., Schlicht M.J., Valyi-Nagy K., Abern M.R., Nonn L. 2021 iScience "Vitamin D sufficiency enhances differentiation of patient-derived prostate epithelial organoids"

Richards Z., McCray T., Marsili J., Zenner M.L., Manlucu J.T., Garcia J., Kajdacsy-Balla A., Murray M., Voisine C., Murphy A.B., Abdulkadir S.A., Prins G.S., Nonn L. 2019, iScience "Prostate stroma maintains the tissue phenotype of human prostate organoids and tumoroids"