NON-CANONICAL ACTIVATION OF HEDGEHOG SIGNALING IN PROSTATE CANCER CELLS IS MEDIATED BY THE INTERACTION OF GLI PROTEINS WITH TRANSCRIPTIONALLY ACTIVE ANDROGEN RECEPTOR

by

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Abstract

The Hedgehog (Hh) pathway is an embryonic development pathway, driven by peptide ligands called hedgehogs. During progression of prostate cancer (PCa), Hh signaling is increased, with especially high activation in castration resistant disease (CRPC). Evidence is lacking for canonical Hh signaling in PCa, indicating the likelihood that non-canonical pathways are involved. Our work shows that transcriptionally active androgen receptor (AR) binding to Gli proteins drives non-canonical Hh signaling in PCa.

Androgen-sensitive LNCaP and androgen-independent LNCaP-AI and LN95 cells were transfected with a Gli-promoter driven luciferase reporter and treated with R1881, enzalutamide, or both, and luciferase activity was measured. Androgen treatment (R1881) induced Gli transcriptional activity while enzalutamide reversed this effect. Similarly, siRNA knockdown of full-length AR (AR-FL) suppressed R1881-induced Gli transcription. Western blot and qPCR confirmed increased expression of endogenous Gli target genes Gli1 and Ptch1 with androgen treatment. Androgen treatment stabilized expression of full-length active Gli3 in a dose-dependent manner but this was reversed by AR knockdown using siRNA. AR binds to Gli3 at the protein processing domain (PPD), which, given the above data, suggests that AR binding to Gli stabilizes full-length Gli3 by preventing phosphorylation and ubiquitination of the PPD, thereby stopping proteolytic cleavage and proteosomal degradation from occurring. Finally, we found that an AR-binding decoy peptide derived from the Gli2 C-terminus can compete with Gli3 for binding to AR, suppressing Gli transcriptional activity in PCa cells.

Our data supports the idea that transcriptionally active AR binding to Gli proteins provides a means for Hh signaling to occur. Not only does AR co-activate Gli transcriptional activity, but it also alters the proteolytic processing of Gli proteins by preventing

phosphorylation and ubiquitination of the Gli PPD by competing with β-TrCP for binding to Gli. Collectively, our findings show the importance of AR-Gli interaction in PCa progression.

Lay Summary

The Hedgehog (Hh) cell signaling pathway is needed for appropriate embryonic development, but it also gets hijacked by cancer cells to promote tumour growth. In prostate cancer, increased activity of this pathway is associated with more advanced disease, indicating that it plays a role in disease progression. My work investigated a novel mechanism by which prostate cancer cells increase Hedgehog pathway activity. We found that androgen receptor (AR) protein, an important hormone receptor in prostate cancer, binds to Gli proteins, the effector proteins of the Hedgehog pathway. Binding of AR to Gli proteins stabilizes them in an active form. Thus androgen receptor protein binding to Gli proteins provides a non-canonical means for activation in prostate cancer cells that might be exploited for new therapeutics to control this disease.

Preface

A version of this thesis has been submitted for publication and is currently in the review process as listed below:

Non-Canonical Activation of Hedgehog in Prostate Cancer Cells Mediated by the Interaction of Transcriptionally Active Androgen Receptor Proteins with Gli3. Na Li, Sarah Truong, Mannan Nouri, Jackson Moore, Nader Al Nakouzi, Amy Anne Lubik, Ralph Buttyan

Dr. Ralph Buttyan was the principal investigator of this study. The experimental design and data interpretation was done by myself, Dr. Na Li, and Dr. Ralph Buttyan, with some input from Mannan Nouri and Jackson Moore. Dr. Na Li trained me with molecular and cellular techniques with which I performed the experiments described in this thesis, with technical support from Dr. Na Li, Mannan Nouri, and Jackson Moore. Dr. Na Li performed some of the experiments found in this thesis, including proximity ligation assay, confocal microscopy, and some cell culture.

The work in submission is supported by a previous study that I participated in, published in *Prostate* (2014) entitled **Determinants of Gli2 Co-Activation of Wildtype and Naturally Truncated Androgen Receptors**.

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List of Abbreviations

aa	Amino acid
ADT	Androgen deprivation therapy
AF-1	Activation function-1
AI	Androgen independent
APC	Antigen-presenting cell
AR	Androgen receptor
ARE	Androgen response element
AR-GBD	Gli-binding domain on androgen receptor
AR-V7	Androgen receptor variant 7
ATCC	American Type Culture Collection
BCC	Basal cell carcinoma
BPH	Benign prostate hyperplasia
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
CRPC	Castration resistant prostate cancer
CS-FBS	Charcoal strip fetal bovine serum
CTD	C-terminal domain
DBD	DNA binding domain
Dhh	Desert hedgehog
DHT	Dihydroxytestosterone
DRE	Digital rectal examination

ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
Gli3-FL	Full-length Gli3
Gli3-R	Repressor Gli3
Gli-A	Activator form of Gli
Gli-DP	Gli2 decoy peptide
Gli-R	Repressor form of Gli
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRE	Gli-response element
GST	Glutathione S Transferase
H&E	Haemotoxylin & eosin
Hh	Hedgehog
HHIP	Hedgehog interacting protein
IHC	Immunohistochemisty
Ihh	Indian hedgehog
KLK	Kallikrein
LBD	Ligand binding domain
LH	Lutenizing hormone
LHRH	Lutenizing hormone-releasing hormone
MB	Medulloblastoma
NLS	Nuclear localization sequence

NTD	N-terminal domain
PBS	Phosphate buffered saline
PCa	Prostate cancer
PPD	Protein processing domain
PSA	Prostate specific antigen
Ptch	Patched
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SAG	Smoothened agonist
scr	Scramble
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
siRNA	Small interfering ribonucleic acid
Smo	Smoothened
Sufu	Suppressor of fused
TAD	Transactivation domain
TAU1/5	Transcription activation unit 1/5
TBS	Tris-buffered saline
TNM	Tumour, node, metastases system
VEGF	Vascular epithelial growth factor

Acknowledgements

I would like to thank my supervisor, Dr. Ralph Buttyan, for the opportunity to pursue graduate studies in his lab. His unwavering support and patience has been a calming presence during my time in the lab, and his insightful advice and constructive criticism have pushed me to become a better researcher and better person. Thank you for being a great mentor and for giving me a wonderful experience throughout this time.

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A huge thank you goes to all the members of the Vancouver Prostate Centre, for helping me through this journey and helping me to make some friends and wonderful memories inside and outside of the lab. I am thankful to all the members of the Buttyan lab, past and present, for making life in the lab interesting and for helping me get through my graduate degree smiling and laughing. There are so many people that have helped me in some way or form during my graduate school journey that I can’t name them all, but know that I appreciate all of you. I am very fortunate to have worked with the incredibly talented and intelligent scientists that I have during my time in the Buttyan lab.

Lastly, I would like to express my gratitude to all my family and friends for their unconditional support and love. I would especially like to thank my parents for instilling in me a passion for learning, and pushing me to take advantage of the opportunities that they never got to have.

Chapter 1: Introduction

1.1 Prostate gland & prostate cancer

1.1.1 Overview

Prostate cancer (PCa) is the most prevalent cancer and the third leading cause of cancer deaths in males in Canada in 2016 [1]. Current statistics predicts that 1 in 7 men will develop prostate cancer, with 21,300 new diagnoses and 4,100 prostate cancer deaths estimated in Canada in 2017 [2]. Despite being a largely indolent disease, it remains a leading cause of cancer deaths, due to a high incidence rate, indicating a need for better screening and better treatment of aggressive disease to reduce mortality rates.

1.1.2 Human prostate gland

The prostate is a small, walnut-sized exocrine gland that is part of the male reproductive system, located below the bladder, surrounding the urethra **(Figure 1.1)**. It functions to produce and store fluid for semen. It is comprised of 3 zones: the transition zone, central zone, and peripheral zone **(Figure 1.2)**. The transition zone is the site of the prostate most prone to developing benign prostate hyperplasia (BPH), surrounding the urethra, lateral to the distal end of the prostate [3]. The central zone is a wedge of glandular tissue found between the transition and periphery zones, while the peripheral zone comprises of the majority of prostate tissue, where about 70% of prostate cancers occur [4].

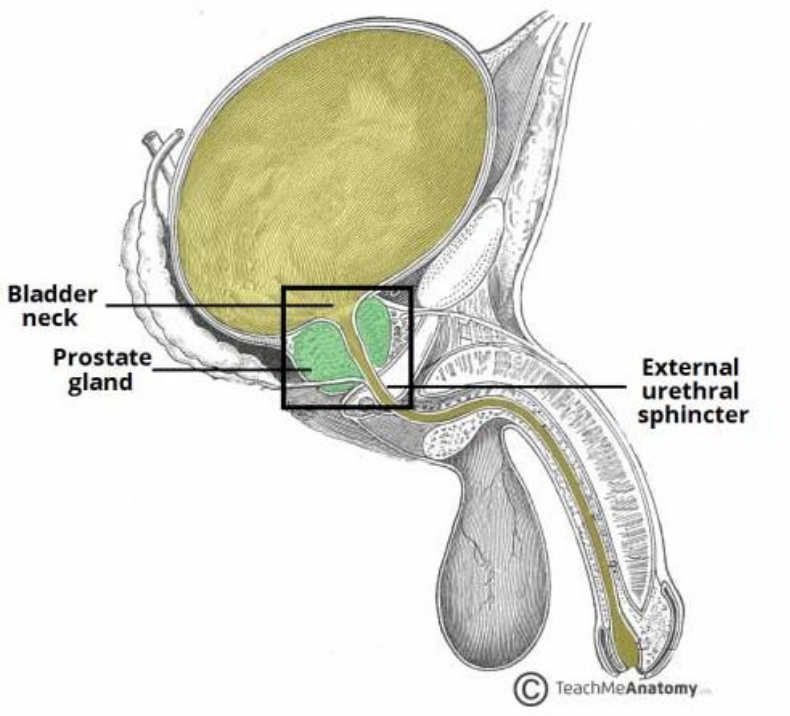

Figure 1.1 Prostate anatomy
Copied from http://teachmeanatomy.info/pelvis/the-male-reproductive-system/prostate-gland/. Accessed October 2017

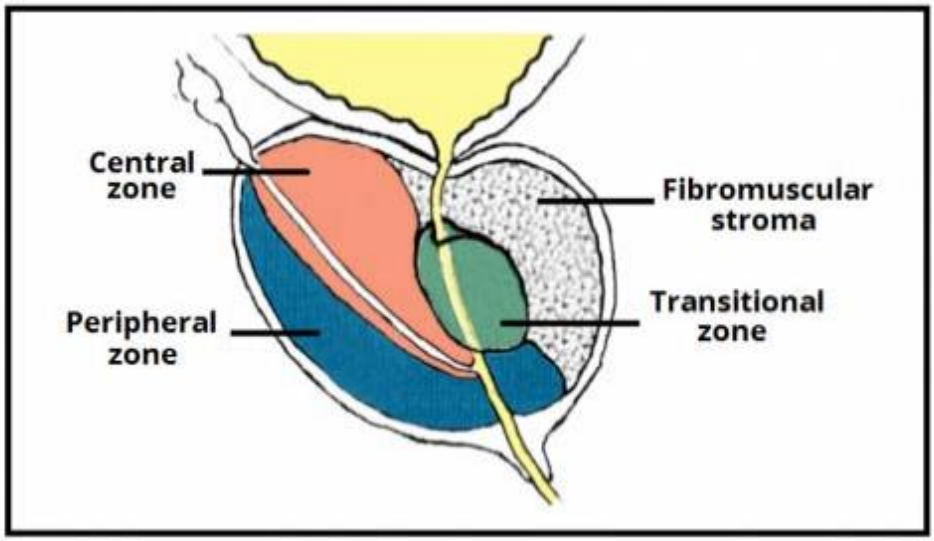

Figure 1.2 Prostate zones
Copied from http://teachmeanatomy.info/pelvis/the-male-reproductive-system/prostate-gland/. Accessed October

2017

Growth of the prostate gland is regulated by activation of the androgen receptor (AR) by male steroid hormones, also known as androgens. AR is a ligand-responsive transcription factor, so binding of androgen to AR regulates genes responsible for development and maturation of the prostate. Secretion of prostate-specific antigen (PSA) is also tightly regulated by AR. Testosterone, made primarily by the testes, is the main androgen found circulating in males, and is converted to dihydroxytestosterone (DHT), a more active androgen, by an enzyme called 5α-reductase in the prostate. Interestingly, in the Dominican Republic, scientists have found a population with high prevalence of 5α-reductase deficiency which causes male pseudohermaphroditism [5]. In many cases, children are born with ambiguous genitalia and consequently raised as girls. With the onset of puberty, spontaneous virilization occurs due to increased serum testosterone concentration [6]. This demonstrates the necessity for 5α-reductase and DHT in proper development of the male genitalia, including the prostate.

1.1.3 Androgen receptor signaling

The androgen receptor is composed of an N-terminal transactivation domain (TAD), a DNA-binding domain (DBD), a hinge domain, and a C-terminal ligand-binding domain (LBD) **(Figure 1.3)**. The N-terminal domain (NTD) is constitutively active, able to activate transcription of AR target genes in LBD-deleted mutants [7]. The NTD contains transcriptional activation function-1 (AF-1), which encompasses 2 transcription activation units, Tau1 and Tau5 [8]. An LKDIL motif within the NTD has been found to mediate activity of Tau1, while a WxxLF motif is responsible for the ligand-independent activity of Tau5 [9].

Figure 1.3 Androgen receptor domains

In the absence of androgens, AR is usually found primarily in the cytoplasm, associated with heat shock proteins [10]. Both testosterone and DHT are able to bind to AR, but DHT has a much higher binding affinity for the receptor, activating AR at much lower concentrations relative to testosterone. Upon binding of testosterone or DHT to AR, a conformation change occurs, resulting in dissociation of heat shock proteins. This facilitates interaction with other co-regulators, including cytoskeleton protein filamin-A [11] and importin-α which bind to a nuclear localization signal (NLS) on AR [12]. This leads to nuclear translocation by AR, where it homodimerizes in a head-to-tail conformation. The conformational change that occurs upon ligand binding to AR causes helices 3, 4, and 12 within the LBD to form the AF-2 binding surface. The AF-2 is a protein-protein interaction surface used by nuclear receptors to bind to the LxxLF-motif of co-activators [13], but in the case of AR, the AF-2 preferentially binds to the FxxLF motif found in the NTD [14]. This results in intramolecular and intermolecular interactions between the NTD and the C-terminal domain (CTD), forming homodimers of AR. Once in the nucleus, AR binds to androgen-response elements (AREs) in the promoter and enhancer regions of its target genes, recruiting other coregulators, thereby modulating gene expression [10]. Androgen receptor signaling not only plays a key role in the development and maturation of the prostate, but aberrant activation of the androgen receptor pathway is a key feature of prostate cancer.

1.1.4 Prostate diseases

The human prostate gland is an inordinate source of health complications in males. Prostatitis is an inflammation of the prostate, estimated to affect up to 30% of males in their lifetime. Prevalent in men aged 35-50 [15], it can be a chronic condition with a poorly

understood etiology. Symptoms include pelvic pain and difficulty in urination [16]. Though often treated with antibiotics, most cases show little evidence for a microbial infection.

Benign prostatic hyperplasia (BPH) is the most common aging-associated condition of the prostate, affecting more than 50% of men by age 60 [17]. It is a benign disease that is associated with an enlargement of the prostate gland due to hyperplasia of stromal and/or epithelial compartments of the prostate. BPH is androgen-driven, with medically-castrated BPH patients showing significant reduction in prostate size and decreased lower urinary tract symptoms [17]. Treatment can involve 5α-reductase inhibitors, in order to reduce DHT levels, but over 25% of patients do not respond to this treatment [17]. Smooth muscle relaxants (alpha-adrenergic receptor antagonists) are also commonly used to relieve lower urinary tract muscle tension that is an important factor in the symptomatic presentation of the disease [18]. Efforts are being made to identify other pathways involved in BPH pathogenesis.

Prostate cancer (PCa) is also a disease of aging. The established risk factors for PCa are age, race, family history [19]. Age is the strongest risk, as studies have shown that incidence of PCa increases after the age of 55, with peaks at ages 70-74, while race is another significant factor. Prostate cancer risk is about 60% higher and mortality is about double in African Americans [19].

At initial development, PCa is dependent on androgenic steroids, only arising in males with intact androgens. Generally, the disease is slow-growing, and can be asymptomatic up until the advanced stage. If detected early, localized disease can be cured by surgery or radiotherapy. Screening for PCa is done by digital rectal exam (DRE) and prostate specific antigen (PSA) blood tests. DRE is used to examine the prostate for asymmetry and the presence of abnormal masses in the gland. PSA is serine protease and a member of the kallikrein-related (KLK) family,

secreted by prostate cells that line the prostate ducts. PSA is positively regulated by AR binding to androgen responsive elements (AREs) located in the promoter and enhancer regions of the gene [20]. Increased PSA serum levels are associated with PCa, and is used as a diagnostic biomarker. Biopsies are suggested for men with abnormal DREs and over 4.0 ng/ml PSA in their blood [21]. Baseline PSA levels can vary among men, so not only is PSA serum concentration taken into consideration, but often change in PSA over time, or PSA velocity, is also considered. There is an increasing number of studies supporting the usefulness of repeated PSA tests to evaluate the kinetics of PSA levels in prostate cancer patients [22]. However, PSA testing is a controversial subject, as there is concern that overtreatment occurs in many cases.

Clinical diagnoses of PCa are accompanied by tumour grading and staging to help determine treatment plans and provide a prognosis for the patient. Tumour grading uses the Gleason system, based on histological arrangement of cancer cells in haematoxylin and eosin (H&E) stained prostate tissue sections. The Gleason scoring system involves using 5 grading categories (Gleason 1-5), determining the most common and second most common grades found in a sample, then combining the two Gleason grades to obtain a Gleason score, ranging from 2-10 [23]. Patients with higher Gleason scores generally have less differentiated tumours and worse prognosis, with increased risk of progression to metastatic disease [24]. Clinical staging is done using the Tumor, Nodes, Metastases (TNM) system, where tumours fall into 4 classes: 1) clinically undetectable, discovered incidentally, 2) palpable tumour confined to the prostate gland, 3) locally invasive tumour, and 4) metastatic disease [25].

Some patients with localized disease (with no identifiable distant metastases) can be simply put under active surveillance (usually patients deemed at low risk upon diagnosis), or can often be cured with surgery or radiotherapy [26]. In cases of advanced disease, or metastatic

prostate cancer, patients are often treated with androgen deprivation therapy (ADT), which depletes systemic androgens, in an effort to inhibit endogenous androgenic signaling. Inhibition of androgenic signaling results in reduction of proliferation of cancer cells and hinders activation of necessary cell survival pathways. ADT is the standard treatment and often highly effective, resulting in size reduction of both primary and metastatic tumours, indicating AR as a key player in PCa progression and growth [25]. Due to the undesirable side effects of constant ADT, studies have looked at intermittent ADT, but it is not currently standard of care.

ADT is practiced with the use of LHRH (lutenizing hormone-releasing hormone) agonists and antagonists, and with anti-androgens. LHRH agonists bind to gonadotropin-releasing hormone (GnRH) receptor, initially stimulate secretion of lutenizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland, both hormones that stimulate testosterone production by the testes. After the initial "tumour flare", continued use of the LHRH agonist causes a sustained reduction in testosterone levels [25]. LHRH antagonists similarly decrease testosterone production, but without the initial tumour flare associated with LHRH agonists. Anti-androgen therapies include androgen receptor antagonists such as bicalutamide or drugs that block androgen synthesis, such as abiraterone, a Cyp17a inhibitor or 5α-reductase inhibitors to prevent the conversion of testosterone to DHT [26]. Unfortunately, many patients with metastatic disease eventually become unresponsive to ADT and develop castration resistant prostate cancer (CRPC).

1.1.5 Castration-resistant prostate cancer

CRPC is a lethal form of PCa, usually with a median survival of 18 to 24 months, although overall survival with CRPC is slowly becoming extended with the development of new drugs and targeted therapies [27]. It usually presents as a recurrent rise in PSA accompanied by

progression of tumour growth, despite low levels of systemic androgens. This advanced form of PCa can occur as either androgen receptor-dependent or androgen receptor-independent, with androgen receptor-dependent CRPC making up the majority of patients.

Androgen receptor-dependent (AR+) CRPC is thought to have 4 mechanisms of becoming resistant to ADT: 1) overexpression of AR that may involve amplification of the AR gene; 2) mutations in AR that encourage promiscuous binding of other steroids, enabling AR activation, 3) intratumoral steroidogenesis, that raises androgen levels within the tumour [28, 29]; and 4) expression of C-terminally truncated ARs (t-ARs) by alternative splicing. These t-ARs are constitutively active. Treatment of newly diagnosed CRPC is changing rapidly. First-line treatment used to involve chemotherapy, especially with docetaxel, a taxane that inhibits microtubule formation [30]. But more recently, potent anti-androgens enzalutamide and abiraterone have been used as front-line therapeutics upon resistance [31]. Many studies have looked at combination therapies that added agents to the docetaxel plus prednisone (a corticosteroid that serves as an immunosuppressant) therapy, but have all failed to show clinically significant benefit [30]. An additional first-line therapy, approved by the FDA in 2010, is Sipuleucel-T, an immunotherapy used to stimulate an immune response in the patient [32]. The therapy involves removing antigen-presenting cells (APCs) from a patient, culturing with a fusion protein that activates the APCs *ex vivo*, and then re-inserting the cells into the patient, inducing an immune response [32]. The efficacy of Sipuleucel-T was evaluated in 3 phase III clinical trials, with each showing improved overall survival and only mild-moderate side effects [32]. For CRPC patients with bone metastases, in 2013 the FDA approved the use of Radium-223, a calcium mimetic that emits alpha particles to the site of bone metastases [33]. In the ALSYMPCA study, it increased overall survival by 3.5 months [34].

In 2010, the FDA approved use of cabazitaxel, a third-generation taxane with a similar mechanism of action as docetaxel, for use as a second-line therapy in patients who failed docetaxel treatment [30]. Cabazitaxel, the first drug to show survival benefit in patients with docetaxel-refractory CRPC, has a lower affinity for the p-glycoprotein drug efflux pump, a common cause of resistance to docetaxel, rendering it a beneficial therapy for advanced CRPC patients [35]. Enzalutamide, an AR inhibitor, was also FDA approved for use as a second-line therapy in docetaxel-refractory CRPC patients, as the AFFIRM trial showed a survival benefit of 4.8 months [36]. In 2014, after the PREVAIL trial, the FDA approval for use of enzalutamide was extended to first-line therapy for metastatic CRPC patients who did not previously have chemotherapy [36]. Despite advances in therapies for CRPC, new and better therapies are needed to extend survival of patients and reduce mortality rates.

1.2 Hedgehog pathway

1.2.1 Hedgehog signaling pathway

The hedgehog (Hh) signaling pathway is a highly conserved signaling pathway that was first characterized in *Drosophila melanogaster*. Mutations linked to the pathway were discovered through a series of genetic screens and the hedgehog pathway was found to be necessary for proper embryonic patterning. In mammals, there are 3 homologous Hh ligands: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). In the absence of hedgehog ligands, Patched receptors (Ptch1/Ptch2) localize to the base of the primary cilium and inhibit movement of transmembrane protein Smoothened (Smo) into the primary cilium **(Figure 1.4)**. Suppressor of Fused (Sufu) is a major negative regulator of the hedgehog pathway as it binds to Gli proteins, the 3 transcription factors and effectors of the Hedgehog pathway (Gli1, Gli2, Gli3) and can alter the ratio of activator:repressor forms of the Gli proteins [37]. Gli1 exists only in a full-length

activator form, while Gli2 and Gli3 occur as in both full-length activator form or cleaved repressor form (Gli3-R) [38]. In the absence of ligand, Gli proteins are sequentially phosphorylated by 3 protein kinases, PKA, CK1, and GSK3β and subsequently processed into repressors [39] or targeted for degradation mediated by the E3 ligase β-TrCP [40] **(Figure 1.5)**.

Upon binding of a hedgehog ligand, Ptch de-represses Smo, which can then move into the primary cilium and alters post-translational processing of the Gli family of transcription factors [41]. Activation of Smo requires not only de-repression by Ptch, but also a second step that is also upregulated by Ptch.; this secondary step is currently not well studied or understood. Active Smo prevents generation and accumulation of the repressor forms of Gli2 and Gli3 [42]. The means by which activation of Smo alters Sufu's negative regulation of Gli proteins is not yet clearly understood.

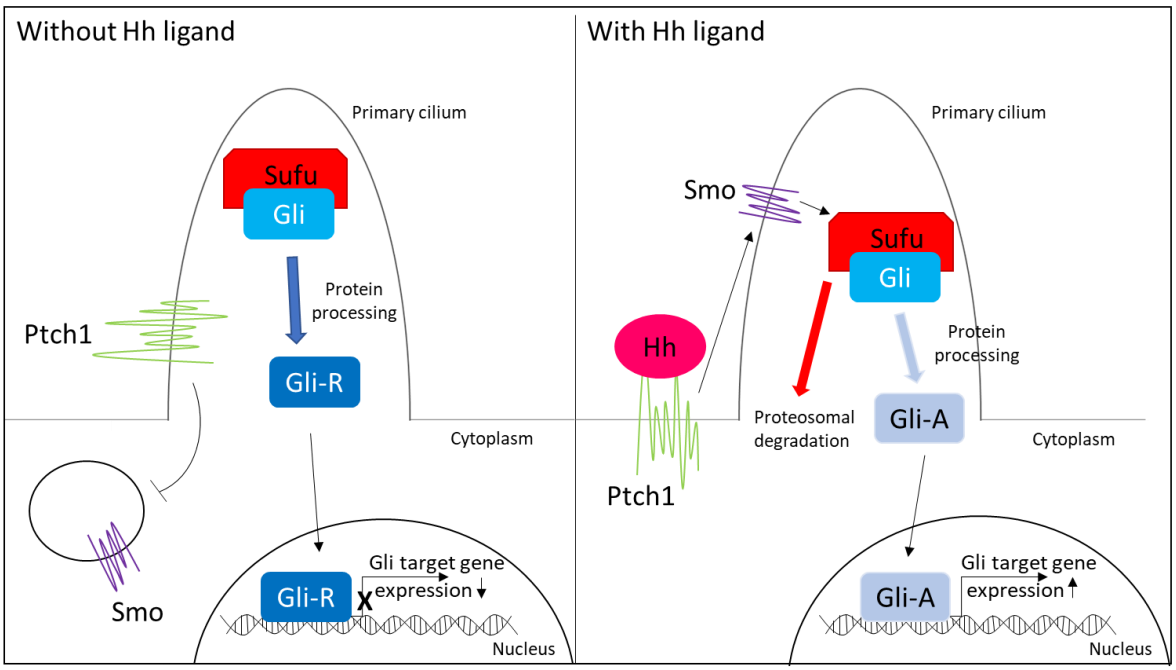

Figure 1.4 Hedgehog pathway signaling
In the absence of Hh ligand, Gli is processed into its repressor form (Gli-R) (left); in the presence of Hh ligand, Gli is processed into its active form (Gli-A) (right)

The activator forms of Gli recognize a Gli-consensus DNA sequence (TGGGTGTC) in promoter and enhancer regions of target genes to activate transcription [43]. One of the target genes of the Hh pathway is Gli1 which is a constitutive activator, thus creating a positive feedback loop. Other target genes include Ptch and hedgehog-interacting protein (HHIP), which sequesters hedgehog ligand, limiting activation of the pathway.

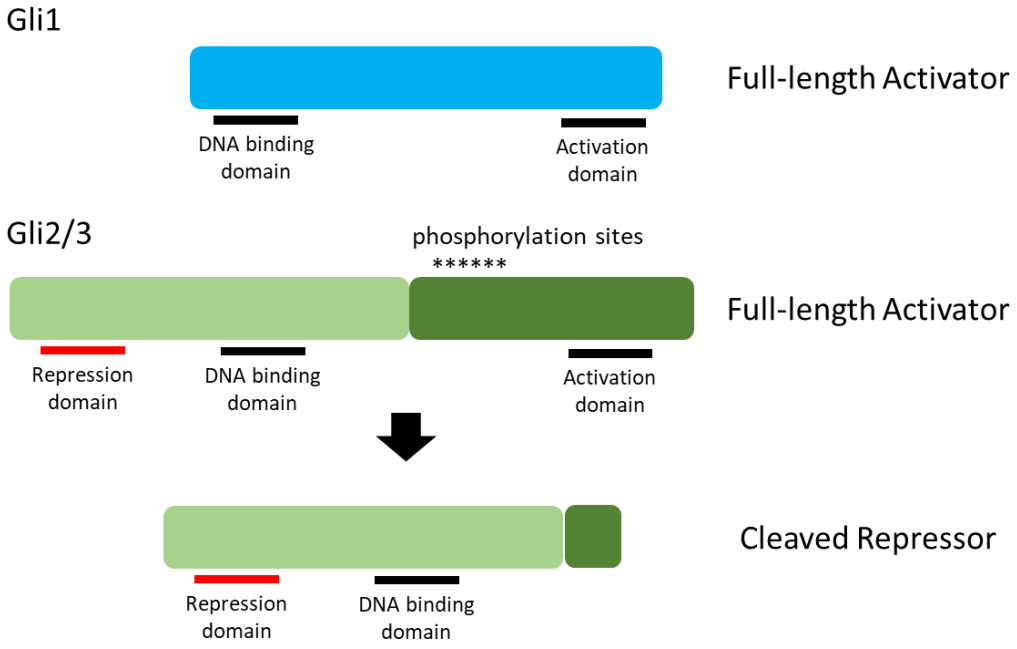

Figure 1.5 Gli protein processing

1.2.2 Biology and function in humans

The hedgehog pathway is most well known as a developmental pathway, involved in numerous developmental processes including cell proliferation, cell differentiation, tissue patterning, and organogenesis. It is needed for normal development of the central nervous system, facial structures, limbs and steroidogenic tissues. The 3 different hedgehog ligands are thought to have similar physiological effects and normal development requires strict control over the timing and gradient of hedgehog ligands. Hh effects are limited by the extent to which the

peptides can diffuse away from the cellular sources in any given tissues. This establishes a gradient and Hedgehog's effects depend on how far potential target cells are from the source.

Hedgehogs are required for various developmental processes. During cerebellar development, Purkinje cells secrete Shh to control proliferation of granule neuron progenitors [44]. In the neural tube, the notochord and floor plate produce Shh, forming a ventral-to-dorsal concentration gradient that dictates which neuronal subtype is formed in which area [42]. Similarly, a posterior-to-anterior concentration gradient is formed in developing vertebrate limbs that regulates limb patterning [44].

In adults, the hedgehog pathway has been found to be involved in the maintenance of stem or progenitor cells in many tissues, thereby regulating tissue homeostasis. It is active in neural stem cells [45], pulmonary epithelium [46], mammary stem/progenitor cells [47], and prostate epithelium [48]. It regulates production of new skin cells [49], and hair follicle growth and cycling [50].

An extremely useful tool for studying the Hh pathway is cyclopamine, an inhibitor of hedgehog signaling. It was first discovered while investigating craniofacial birth defects in lambs in the United States [51]. Pregnant ewes grazing on *Veratum californicum* (corn lily) gave birth to lambs with cyclopean type birth defects. It is now known that corn lilies contain cyclopamine, a steroidal alkaloid that binds to Smo, inhibiting hedgehog signaling [52].

1.2.3 Hedgehog pathway in cancer

Considering the critical role of Hh in embryonic development, it is no surprise that cancer cells can exploit the pathway to promote tumour cell proliferation. An oncogenic role for the hedgehog pathway has been established in a number of cancers. Oncogenic hedgehog signaling was first discovered in patients with Gorlin syndrome, a hereditary condition where patients

develop several basal cell carcinomas (BCCs) and medulloblastomas (MBs) in their lifetime. Ptch and Sufu mutations underlie predisposition to Gorlin syndrome [53]. Mice with haploinsufficiency for Ptch develop tumours with the characteristics of basal cell carcinoma [54] and medulloblastoma [55, 56]. Gain-of-function mutations in Smo have also been associated with basal cell carcinomas [57]. Activated hedgehog has also been demonstrated in many other cancers, including ovarian [58], breast [59], pancreatic [60], colon [61], lung [62], and prostate cancer [63]. There is much interest in the role that the hedgehog pathway and Gli proteins play in cancer, but studying the pathway is more complicated due to the existence of alternate pathways of Gli activation. These include activation of Gli expression by TGFβ [64], β-catenin [65], and RAS/RAF/MEK/ERK[66] -mediated signaling.

Three main modalities of Hh pathway activation have been suggested: ligand-independent signaling, ligand-dependent autocrine signaling, and ligand-dependent paracrine signaling. These 3 modalities are not mutually exclusive, but can occur concurrently. Ligand-independent signaling can occur as described above, where loss-of-function mutations in Ptch or Sufu, or gain-of-function mutations in Shh, Gli, or Smo, can cause aberrant signaling as occurs in BCC and MB. It is believed that ligand-dependent signaling is the cause of aberrant signaling in other tumor types. Evidence for association of hedgehog signaling with cancer includes previous reports of increased hedgehog ligand and target gene expression, as well as findings that inhibition of hedgehog/Gli by cyclopamine or Gli knockdown suppresses cell growth *in vivo* and *in vitro* [67, 68]; these reports support the argument for autocrine signaling.

Evidence has emerged in several cancers in which hedgehog ligands are produced by tumour cells and signal the surrounding stroma to produce growth factors. A variation of this has also been found in B-cell malignancies [69] and prostate cancer [28], where hedgehog ligand is

secreted by surrounding cells to stimulate growth of tumour cells. One of the first studies showing ligand-dependent paracrine Hh signaling was done by Bushman and colleagues in prostate cancer [70]. In this study, stromal Gli1 expression was induced by expression of Shh in LNCaP xenograft mice. Growth rates of Shh overexpressing tumours and control tumours were comparable *in vitro*, but were increased *in vivo.* This difference is attributed to a growth-promoting effect of the stroma *in vivo*. Quantitative RT-PCR and radioactive in situ hybridization were used to confirm that Hh levels in the tumours correlate to murine Gli1 and Ptch1 expression levels, but expression of human Gli1 and Ptch1 were absent or barely detectable [70]. Similar results have been recapitulated in subsets of colorectal, endometrial, ovarian, and pancreatic cancers [71]. Paracrine Hh signaling was observed in co-cultures of pancreatic tumour cells with fibroblasts transfected with a Gli luciferase reporter, where induction of Gli reporter activity correlated with production levels of Hh ligand by tumour cells [71]. Additionally, there was a correlation between stromal-derived Gli1 and Ptch1 mRNA and tumour-derived Hh ligands levels in primary human tumour xenografts [71].

Primary cilia play an important role in normal hedgehog signaling. Primary cilia are microtubule-based organelles that extrude from the cell surface, acting as sensors and coordinating cellular signaling pathways. Tumour cells are often ciliated in BCC, and studies have shown that inhibition of ciliogenesis slows Smo-induced cell growth, but can also accelerate growth of tumours induced by activated Gli2 [72]. The paradoxical role of primary cilia in cancer is further complicated by the lack of these organelles in many tumour cells. Ciliogenesis usually occurs in growth-arrested cells, while cancer cells are fast growing and are not often ciliated.

1.2.4 Hedgehog pathway in prostate cancer

Hedgehog is involved in development of the prostate, as well as regeneration of prostate epithelial cells in adults. Hedgehog ligands have been found to be overexpressed in prostate cancer, in human samples, cell lines, and animal models. Loss-of-function mutations of Sufu are the only component of the hedgehog pathway to have been found mutated thus far, although this is a rare occurrence and is not found in most prostate cancer tumours [73]. BCCs and medulloblastomas often contains mutations in Ptch or Smo, but no similar abnormalities have been reported for human prostate tumours or prostate cancer cell lines.

General consensus is that increased hedgehog signaling in PCa is hedgehog ligand-dependent, mediated in both paracrine and autocrine manners. Early studies show suppression of PCa cell growth *in vitro* and *in vivo* using cyclopamine (a Smo antagonist) or anti-Shh antibodies, which supports autocrine signaling [67]. However, these studies generally use relatively high doses of the Smo-antagonist, cyclopamine, which has demonstrated off-target effects. Cyclopamine fails to inhibit hedgehog target gene expression (Ptch1, Gli1, and Hh reporter) in LNCaP, PC3, and 22RV1 prostate cancer cell lines [74]. Additionally, exogenous overexpression of mutated (constitutive active) Smo in transgenic mouse prostates did not induce any morphogenic or cellular changes in the prostate, although it was able to induce rhabdomyosarcoma, BCC, medulloblastoma and pancreatic lesions [75]. Gli2 knockdown by anti-sense oligonucleotide in PC3 cells increased apoptosis and decreased cell growth, accompanied by enhanced chemosensitivity to paclitaxel *in vitro* and *in vivo* [68].

During prostate development, Hh signaling occurs through epithelial-stromal signaling and PCa may recapitulate this process. This idea is supported by the study mentioned above by Bushman and colleagues, showing that Gli1 localized to the stromal compartment while prostate

epithelium expressed Shh in LNCaP-derived tumour xenografts [70]. Not only this, but Shh expression by LNCaP cells increased Gli expression in the stroma and overexpression of Shh significantly increased tumour growth [70].

There have been several studies that confirm a role for active Hh pathway signaling in human PCa tissues. One study involving 239 prostate carcinomas, 15 precancerous high-grade neoplasias, and 135 benign prostate tissues found higher Shh expression more often in tumours than normal adjacent tissue, as well as a correlation between higher Shh levels and Ki67, a proliferation marker [67].

Not only is Hh signaling active in PCa, but various studies have shown that there is a correlation between increased Hh pathway activation and advanced PCa. Increased HHIP and Ptch1 expression were found in PCa specimens and correlated with high Gleason score and presence of metastases [73]. Epithelial Ptch expression in tumour tissue is higher in metastases than primary tumours, and correlated with Ki67 and vascular epithelial growth factor (VEGF), a potent angiogenic factor [76]. A study involving 231 hormone naïve PCa samples found a significant correlation between Shh expression and Gleason score [77]. Expression of Gli2 was found to be significantly higher than in benign prostate hyperplasia samples [68]. Kim et al. produced retrospective study of 155 PCa samples in which they looked at protein expression of Shh, Ptch, Smo, Gli1 and Sufu by IHC and correlated them with clinicopathological parameters. Shh was found to be correlated with tumour size, while Shh, Ptch, Smo, and Gli1 were all found to be correlated with Gleason score and pretreatment PSA levels [78]. Additionally, multivariate analysis found Shh to be an independent prognostic factor of PSA recurrence [78]. Hedgehog signaling has been found to have a role in metastasis, as expression of Gli1 into a poorly metastatic cell line (AT2.1) significantly increased the metastatic ability of the cells [48].

1.2.5 Cross-talk between androgen and Hedgehog signaling pathways

There is a bidirectional relationship between androgen signaling and the Hh signaling pathway in PCa that may affect the development of castration resistant disease. Androgen deprivation of AR+ androgen growth-dependent PCa cells increased expression of Shh mRNA, by 30,000-fold, as well as Ihh and Dhh, although to a smaller extent [79]. Upregulated expression of Shh mRNA and protein in androgen-deprived medium was accompanied by similar increases of Shh in culture medium. This Shh-conditioned medium was functional as an inducer of Gli activity in mouse fibroblasts. [79]. In addition to upregulation of Shh, androgen ablation of prostate cancer cell lines also shows increased expression of Gli2 and Ptch1 mRNA, supporting the idea that aberrant hedgehog signaling contributes to PCa progression [79]. Induction of Hh signaling by androgen ablation occurs not only in prostate cancer cell lines, but also in human prostate tumour specimens. Efstathiou et al. report that four-month exposure to androgen deprivation therapy, alone or in combination with chemotherapy, induces increased expression of various Hh signaling components (Shh, Smo, Gli1, and Gli2) [80].

Not only does androgen signaling affect the Hh signaling pathway, but the reverse is also true. In LNCaP and other prostate cancer cell lines, siRNA knockdown of Gli2 and treatment with Gli inhibitors GANT61 and GANT58 also downregulated androgen-regulated genes [81]. Conversely, exogenous overexpression of Gli1 or Gli2 increased expression of androgen-regulated genes and enabled androgen-independent growth of prostate cancer cells [63]. While co-immunoprecipitation studies showed that Gli proteins (1, 2 and 3) are associated with AR in PCa cells, GST-pulldown assays established that they are direct binding partners [82]. The AR-binding domain on Gli2 was mapped to amino acids (aa) 628-897, and it was determined that at least part of the C-terminal TAD was also required for co-activation of AR signaling [82]. The

Gli-binding domain on AR was also mapped by GST-pulldown and localized to a region encompassing the core sequence of the Tau5 transactivation domain (WxxLF) within the AR N-terminus ($aa_{392-558}$). Tau5 is sometimes referred to as the N-terminal "ligand-independent activation domain", as it has been implicated in AR signaling in the absence of androgens [83]. Interestingly, Gli2-FL and the Gli2-CTD were both found to also co-activate constitutively-active truncated ARs (AR-V7 and AR567es), but this is not surprising since both contain the Tau5 domain [82]. ChIP analysis of Gli2 overexpressing LNCaP cells showed that Gli2 was associated with androgen response elements (AREs) near androgen responsive genes, PSA and PGC (progastricsin or pepsinogen C), confirming co-activation of AR by Gli2. Conversely, the same ChIP study showed that AR was bound to Gli-response element (GRE) near the Ptch1 gene [82].

1.3 Hypothesis

Data from the ChIP experiment mentioned above in which AR was found bound to Gli2 at the GRE of the Ptch1 gene indicated the possibility that not only does Gli mediate AR signaling, but the inverse is also true and AR co-activates Gli in prostate cancer cells. Data from an online ChIP database, the Cistrome project, similarly shows accumulation of AR at the GRE of the Gli1 gene in two AR ChIP experiments, both in VCaP cells **(Figure 1.6)**.

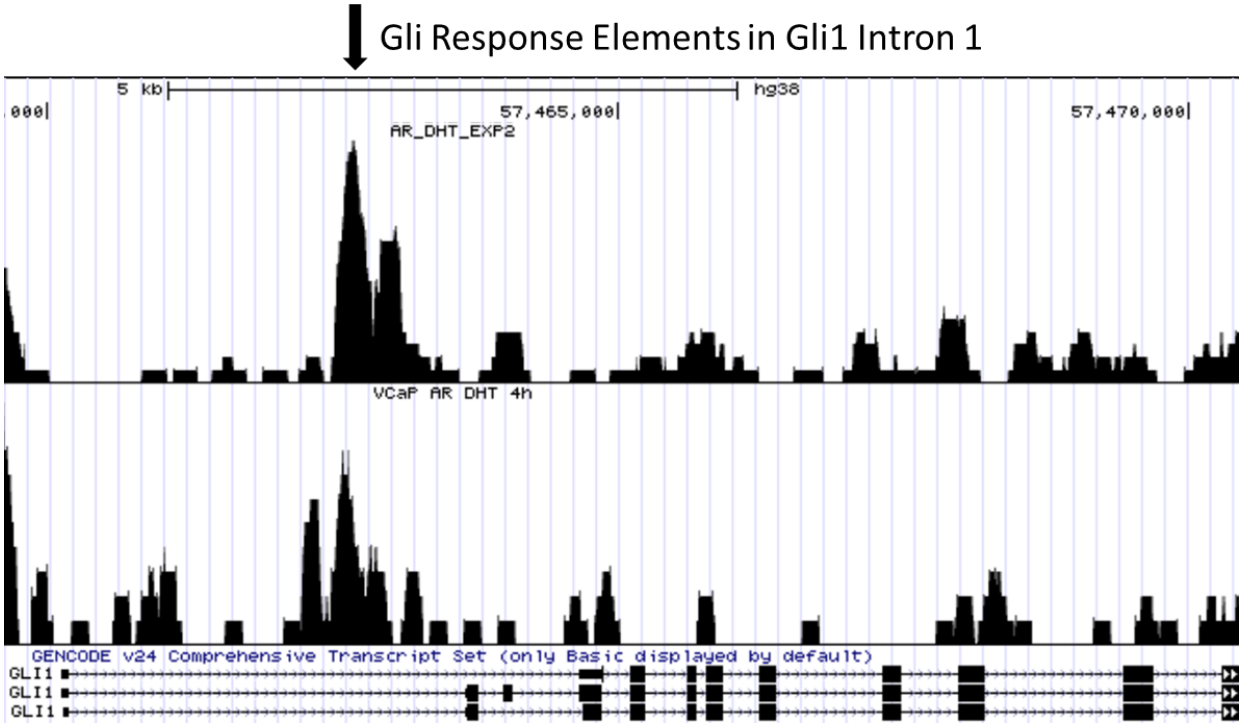

Figure 1.6 AR ChIP data in VCaP cells from the Cistrome Project

AR ChIP data from the Cistrome Project, an online database, shows accumulation of AR at the Gli-response element of the Gli1 gene in VCaP cells (Top: Asangani IA et al. 2014 [84] and bottom: Cai C et al. 2012 [85]). Black bars represent the number of amplification reads during sequencing of ChIP DNA.

Since it has been established that AR and Gli directly bind to each other and that AR binds to GREs of Hh pathway target genes, we hypothesized that AR is a co-activator of Gli and mediates non-canonical hedgehog signaling in prostate cancer.

Chapter 2: Materials and methods

2.1 Plasmid generation and constructs

Human AR-FL (906 amino acids long) was provided by Dr. Paul Rennie (Vancouver Prostate Centre) and AR-V7 was from Dr. Jun Luo (Johns Hopkins Medical Center). The vector expressing Gli3-FL was previously described [82]. Gli luciferase reporter was generated by subcloning an 8X repeat Gli binding consensus sequence into KpnI and BglII sites of pGL4.28 (Promega, Inc., Madison, WI); the vector was validated by sequencing. Human glucocorticoid receptor beta (GR-FL) and human estrogen receptor alpha (ER-FL) was also provided by Dr. Paul Rennie (Vancouver Prostate Centre).

Coding sequences of GST-tagged Gli2 fusion-polypeptides, aa628-897, 628-836, 628-805, 764-836 and mutant 628-897 were subcloned into EcoRI and SalI sites of pGEX-5X-3 vector (Clonetech, Mountain View, CA). QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was utilized to introduce Aspartate (D) for Arginine (R) residues of the respective Gl2 phosphorylation site [86]. Mutagenesis primers used were as follows: amino acid 821R/D and 822R/D, 5′- C ACC GTG AGC GAC GAC TCC TCC GGC ATC TC -3’; amino acid 833R/D and 834R/D, 5’- TAC TTC TCC AGC GAC GAC TCC AGC GAG GCC -3′; amino acid 864R/D and 865R/D, 5’- ACG GAC GCG TCG GAC GAC TCG AGC GAG GCC -3’.

A Gli2 decoy peptide was generated by subcloning N-terminal myc-tagged Gli2aa$_{628\text{-}897}$ into EcoRI and XbaI sites of pLenti4-TO (Thermo Fisher), followed by an in-frame C-terminal 3X repeats of a nuclear localization sequence (NLS; forward, 5’- CT AGA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA TAG T -3’, reverse, 5’- CT AGA CTA TAC CTT TCT CTT CTT

TTT TGG ATC TAC CTT TCT CTT CTT TTT TGG ATC TAC CTT TCT CTT CTT TTT TGG ATC T -3', inserted into XbaI sites.

2.2 Cell culture

Human prostate carcinoma cell lines LNCaP, CWR22rv1, PC3, and VCaP, as well as mouse preosteoblast cell line MC3T3-E1 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and human embryonic kidney cell line 293FT was obtained from Thermo Fisher. LN-AI [63] and LN95 (from Dr. Stephen Plymate, University of Washington) cell lines are androgen-independent derivatives of the LNCaP cell line. R1-D567 and R1-J cells are CWR1-dereived cell lines, received from Dr. Scott Dehm (University of Minnesota). LAPC4 cells were received from Dr. Charles Sawyer, MSKCC and C42 cells are from the Vancouver Prostate Centre.

LNCaP, CWR22rv1, PC3, C42 and R1-J cells were cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin/streptomycin, while LN-AI, LN95, and R1-D4567 cell lines were cultured in RPMI1641 medium supplemented with 10% charcoal-stripped serum (CS-FBS) (GE Life Sciences, Mississauga, ON, Canada) and penicillin/streptomycin. VCaP cells were cultured in Dulbecco's Modified Eagle's Medium (Hyclone), supplemented with 10% FBS and penicillin/streptomycin. MC3T3-E1 cells were cultured in Alpha Minimum Essential Medium (Hyclone) supplemented with 10% FBS and penicillin/streptomycin. 293-FT cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. LNCaP cells were authenticated by short tandem repeat profiling (IDEXX, Inc., Markham, ON, Canada).

2.3 Transfection and transduction

2.3.1 Small interfering RNAs

Pre-validated small interfering RNA (siRNA) targeting full length AR (AR Silencer Select, Thermo Fisher, S1539) or non-targeting negative control siRNA (Silencer Select Negative Control no.1, Thermo Fisher) were transfected into LNCaP, LN-AI and LN95 cells with Lipofectamine RNAiMAX (Thermo Fisher) using reverse transfection. siRNA was prepared according to manufacturer procedures, added to cells in suspension and then plated onto appropriate cell culture dishes.

2.3.2 Plasmid DNA

2.3.2.1 Transfection

For co-immunoprecipitation studies and luciferase reporter assays plasmids were transiently co-transfected into PCa or 293FT cells using lipofectamine 3000 (Thermo Fisher) using manufacturer's protocols.

2.3.2.2 Transduction

Second generation lentivirus was produced by transient co-transfection of a 10-cm plate of $4x10^6$ 293FT cells with 10 µg psPAX2, 5 µg pMD.2G VSVG, and 15 µg of pLenti4-TO or pLenti4-TO_myc-$Gli2_{628\text{-}897\_3xNLS}$ (Profection Mammalian Transfection System, Promega). Lentivirus was collected, filtered through a 0.45 µm filter, aliquoted and stored at -80°C.

For lentiviral transduction, $1x10^5$ cells/well were seeded in a 6-well tissue culture plate and infected the following day with lentivirus. Medium was changed regularly and the appropriate antibiotic for selection was added to the culture medium 1 week after transduction, with cells maintained in antibiotic-containing medium thereafter.

For generation of doxycycline-inducible LNCaP-AI-myc-Gli2$_{628\text{-}897_3xNLS}$, two steps of lentiviral transduction were performed. Stable LNCaP-AI cells expressing the Tet-Repressor (TR) were generated by transducing lentivirus (pLenti6/TR vector, Thermo Fisher) with the ViraPower™ Lentiviral Packaging Mix (Thermo Fisher). Cells expressing TR were selected for using 10 µg/ml blasticidin and subsequently transduced with lentivirus made from either pLenti4-TO (empty Control) or pLenti4-TO_myc-Gli2$_{628\text{-}897_3xNLS}$ and were selected for with 100 µg/ml zeocin.

2.4 Luciferase assay

LNCaP, LN-AI, LN95 and R1-d567 cells were transiently co-transfected with Gli-luciferase reporter (pGL4.28 Gli-luc), pCMV-EGFP, and control or AR-V7 vector. Cells were washed with PBS, lysed with passive lysis buffer and luciferase activity of cell lysate was measured using the Dual-Luciferase Reporter Assay System (Promega) on the Tecan Infinite 200 Pro (Tecan Group Ltd., Männedorf, Switzerland), according to manufacturer's protocol. GFP readings were taken prior to the luciferase assay and were used as transfection controls in the analysis of luciferase assay data (luciferase values were normalized to GFP).

2.5 Real-time PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher) following manufacturer's protocols. 1-2 µg of total RNA was used to synthesize cDNA using random hexamers (Thermo Fisher) and M-MLV reverse transcriptase (Thermo Fisher) according to manufacturer's protocols. Quantitative real-time PCR (qRT-PCR) was performed using the following primer pairs: PSA, KLK2, Gli1, Ptch1, and 18s with FastStart Universal SYBR Green PCR Master Mix (Roche Diagnostics, Laval, QC, Canada) on the ViiA7 Real-Time PCR System (Applied Biosciences, Streetsville, ON, Canada). qRT-PCR cycle conditions were as follows: 2

minutes at 50°C followed by 10 minutes at 95°C, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. 18s gene expression was used as an internal control and relative fold changes were calculated using the $2^{(-\Delta\Delta CT)}$ method. Melt curves were generated for each primer set at the end of each qRT-PCR to ascertain the specificity of the reaction.

Table 2.1 qRT-PCR primer sequences

Gene name	**Primer sequences**
18s	F: TTG ACG GAA GGG CAC CAC CAG R: GCA CCA CCA CCC ACG GAA TCG
PSA	F: AGT GCG AGA AGC ATT CCC AAC R: CCA GCA AGA TCA CGC TTT TGT T
KLK2	F: GCT GCC CAT TGC CTA AAG AAG R: TGG GAA GCT GTG GCT GAC A
Gli1	F: GGC TCG CCA TAG CTA CTG AT R: CCA GCG CCC AGA CAG AG
Gli2	F: CCC CTA CCG ATT GAC ATG CG R: ACA GAA TGA GGC TCG TAA TGG T
Gli3	F: CGA ACA GAT GTG AGC GAG AA R: GGC TGC ATA GTG ATT GCG T
Ptch1	F: TCT CCA ATC TTC TGG CGA GT R: TGG GAT TAA AAG CAG CGA AC

2.6 Western blotting

After being washed with PBS, scraped and pelleted by centrifugation, cells were lysed with 1% NP-40 lysis buffer containing 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM iodoacetamide, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics). After a 30-minute incubation on ice followed by centrifugation at 12,000 rpm for 10 minutes, supernatant containing cell lysates were collected.

Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher). Equal amounts of protein (30-100 µg) were denatured at 100°C for 4 minutes with sodium dodecyl sulfate (SDS) -containing loading buffer. Samples were loaded onto a 4-15% gradient SDS polyacrylamide gel and separated by electrophoresis, then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using the wet-transfer method (20 V overnight at room temperature). After transfer, the membranes were blocked with 5% skim milk in tris-buffered saline (TBS, 50 mM Tris/pH 7.5, 0.15 M NaCl) for 1 hour, then immunoblotted with primary antibody diluted in 5% skim milk in TBS-Tween (TBS, 0.05% Tween-20) overnight at 4°C. After 3x 5-minute washes, membranes were incubated in the appropriate secondary antibody (mouse/rabbit IgG-HRP 1:2000 from Santa Cruz, Dallas, TX, USA or mouse/rabbit 680/800 1:5000 from LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature. 3x 5-minute washes were done, then membranes were imaged with the Odyssey scanned (LI-COR Biosciences) or detected with Pierce ECL Western Blotting Substrate (Thermo Fisher) and imaged using autoradiography films (Genessee Scientific, San Diego, CA, USA) fixed/developed with a film processor (EL-Rad Services, Vancouver, BC, Canada).

2.7 Antibodies and reagents

Mouse anti-human AR-441 (sc-7305) antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA); rabbit anti-human Gli1 (#2553), PSA (#2475), β-TrCP (#4394), and GAPDH (#5174) antibodies were from Cell Signaling (Cell Signaling Technologies, Danvers, MA, USA); rabbit anti-human Gli3 (GTX104362) antibody was from GeneTex (Genetex Inc., Irvine, CA, USA); mouse anti-Vinculin (V4505) antibody was from Sigma-Aldrich (Sigma Aldrich, Oakville, ON, Canada), rabbit anti-human Ptch1 (ab39266) was from Abcam (Abcam,

Cambridge, MA, USA), and mouse anti-myc antibody was from EMD Millipore (Etobicoke, ON, Canada).

2.8 Enzalutamide was purchased from Chemexpress Co., Ltd (Shanghai, P.R. China). R1881 was from Perkin Elmer (Shelton, CT, USA) and doxycycline was from Sigma Aldrich. Smoothened agonist, SAG (N-Methyl-N′-(3-pyridinylbenzyl)-N′-(3-chlorobenzo[b]thiophene-2-carbonyl)-1,4-diaminocyclohexane), was purchased from Calbiochem (Etobicoke, ON, Canada).

2.9 Co-immunoprecipitation

For co-immunoprecipitation, cell lysates were prepared in the same manner as described above for western blot. After obtaining protein concentrations, 1.5-3 mg of whole cell lysates were pre-cleared with isotype matching IgG for 1 hour at room temperature. Cell lysates were then incubated with 4-6 µg per mg of lysate of mouse anti-AR/rabbit anti-Gli3 antibody overnight at 4°C on a rotator. Immune complexes were precipitated with 30 µl of a 50% slurry of Protein A/G agarose beads (Thermo Fisher) for 4 hours at 4°C on a rotator. The mixed beads procedure ensures better capture of different antibody types when using polyclonal antibodies. SDS containing loading buffer was added to samples and were heated at 100°C for 4 minutes to elute immunoprecipitates from agarose beads and denature proteins. 30-50 µg of cell lysates were used as input samples while running 4-15% gradient SDS polyacrylamide gels with the immunoprecipitates. Samples were then transferred onto PVDF membrane and immunoblotted with the appropriate antibodies following the same protocol as detailed above for western blot.

2.10 Proximity ligation *in situ* assay and confocal microscopy

Proximity ligation assay (PLA) was performed with Duolink® In Situ Detection Reagent Fluorescence Kit (Sigma-Aldrich). Cells were grown on glass coverslips and fixed with 4% PFA for 30 minutes, followed by permeabilization with 0.5% Triton-X in PBS for 5 minutes. Cells

were blocked with manufacturer-provided blocking solution for 30 minutes at 37°C and incubated overnight at 4°C in a humidified chamber with a mixture of rabbit anti-Gli3 and mouse anti-AR antibodies. Samples were then incubated for 1 hour at 37°C with the corresponding PLA probes (anti-rabbit Plus and anti-mouse Minus, provided in kit) conjugated to specific oligonucleotides. The PLA oligonucleotides were hybridized and circularized by ligation (30 minutes at 37°C) and amplification (100 minutes at 37°C). Visualization of PLA signals was on a Zeiss LSM 780 Confocal Laser Scanning Microscope (Carl Zeiss, Germany). Quantification of staining was done with Duolink® ImageTool softwear (Sigma-Aldrich).

2.11 GST pull-down assays

GST-tagged Gli2 polypeptides were isolated from transfected E coli. BL21 (DE3) pLysS cells (Promega, Inc) using glutathione-sepharose 4B beads (Amersham, Piscataway, NJ) and were visualized after SDS-PAGE with Coomassie blue staining. Human AR truncates (aa_{1-558}, $aa_{392-558}$) or βTrCP (p4489 Flag-βTrCP, Addgene #10865) proteins were prepared using the Quick Coupled T7 TNT in vitro transcription /translation kit (Promega) in the presence of methionine. These proteins were pre-cleared with GST-bound glutathione beads prior to incubation with GST-tagged recombinant proteins. Immune complexes captured on glutathione beads were eluted for SDS-PAGE followed by western blot analysis with appropriate antibodies. For controls, in vitro translated AR truncates and β-TrCP proteins were incubated with GST beads alone and eluates were analyzed as above.

2.12 Statistical Analysis

Analysis of data was done using Student t-test to compare control and treatment groups; p-values less than 0.05 were considered statistically significant as follows: *P< 0.05, **P< 0.01, ***P< 0.001.

Chapter 3: Results

3.1 Transcriptionally active AR upregulates Gli transcriptional activity in PCa cells

3.1.1 The effect of transcriptionally active AR on a Gli-luciferase reporter

We first wanted to look at whether AR has an effect on transcriptional activity of Gli. Using a luciferase reporter vector containing the Gli-consensus binding sequence in the promoter, we tested whether androgen (R1881) affected Gli transcriptional activity in 5 AR+ PCa cell lines (parental LNCaP, androgen growth-independent LNCaP-AI and LN95, LAPC4, and R1-D567, a CWR1 variant that expresses only a C-terminal truncated AR (AR-V567es)). LNCaP and its variants, along with LAPC4 showed upregulation of Gli-luciferase reporter expression with the addition of R1881, an effect that was blocked by the addition of enzalutamide (ENZ), an AR antagonist **(Figure 3.1)**. While R1881 and enzalutamide showed no effect on R1-D567 cells, the cell line has a higher basal level of Gli reporter activity relative to AR-FL expressing cell lines.

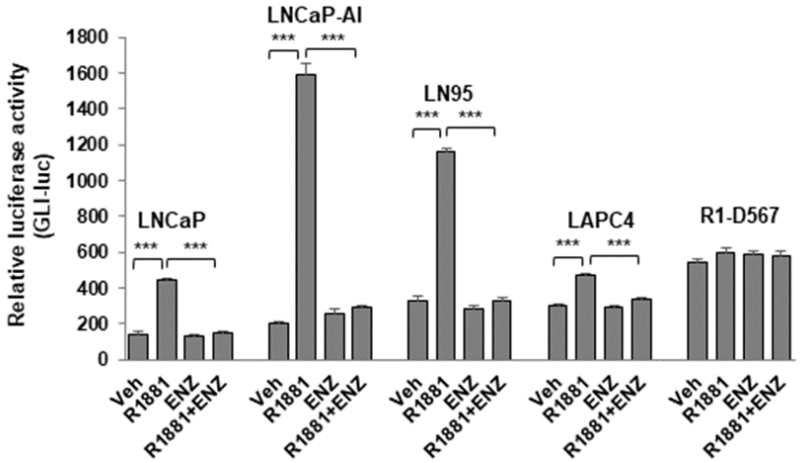

Figure 3.1 Androgen upregulates Gli-luciferase reporter activity in AR+ PCa cell lines and enzalutamide blocks this upregulation

Relative activity of a Gli-luciferase reporter assessed by luciferase assay in 5 PCa cell lines comparing cells in the presence of vehicle (Veh), R1881 (1 nM), enzalutamide (Enz, 10 µM), or both for 48 hours. Bars represent mean± SD, **P*<0.05; ****P*<0.001; ns, not significant.

3.1.2 Androgen-induced Gli transcriptional activity is blocked by knockdown of AR

In order to show that the effects of androgen are mediated by the androgen receptor, we tested whether knockdown of AR affects androgen-induced Gli activity. Transfection of siRNA targeting full-length AR into LNCaP and androgen growth-independent LNCaP derivatives LNCaP-AI and LN95 blocked upregulation of Gli-luciferase reporter activity induced by androgen.

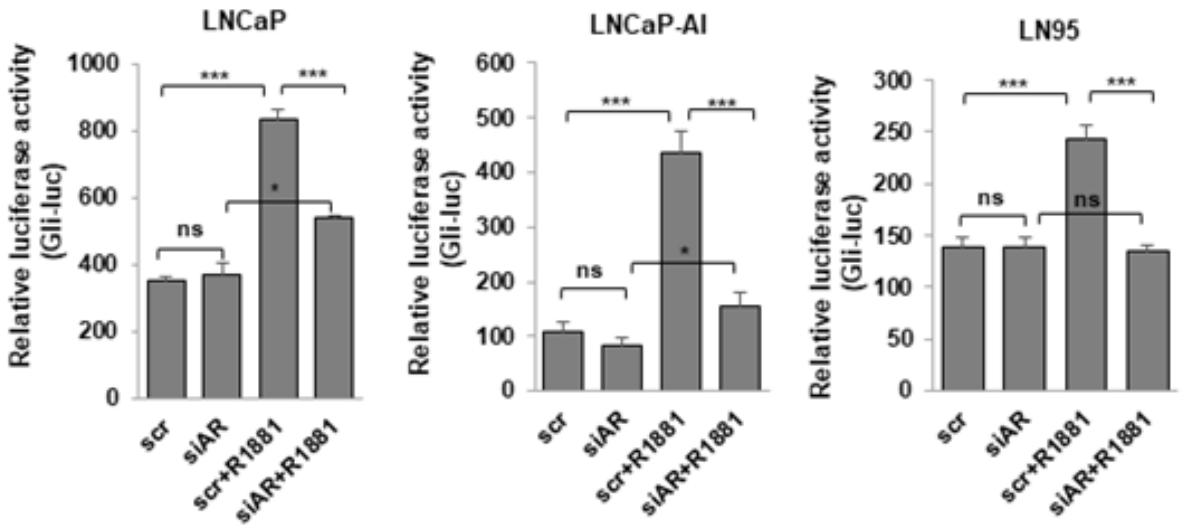

Figure 3.2 Knockdown of full-length AR inhibits upregulation of Gli-luciferase reporter activity by androgen

Relative Gli-luciferase reporter activity measured by luciferase assay in LNCaP, LNCaP-AI and LN95 cells transfected with scramble (scr) or siRNA against full-length AR (siAR), followed by treatment with R1881 (1 nM) for 48 hours. Bars represent mean± SD, *P<0.05; ***P<0.001; ns, not significant.

3.1.3 AR-V7 overexpression upregulates Gli-luciferase reporter

Since AR-V7 is a constitutively active truncated androgen receptor, we also looked at the ability of AR-V7 to induce Gli transcriptional activity. Transfection of AR-V7, a constitutively active AR truncated variant, into AR+ (LNCaP) or AR- (PC3) cells also upregulated Gli-luciferase reporter activity, with and without androgens **(Figure 3.3)**. siRNA knockdown of AR-FL further enhanced the ability of AR-V7 to induce Gli reporter in the presence and absence of androgens (in AR-V7 overexpressing LNCaP cells, scr vs. siAR without R1881 P=0.0008, in the presence of R1881 scr vs. siAR P=0.00105).

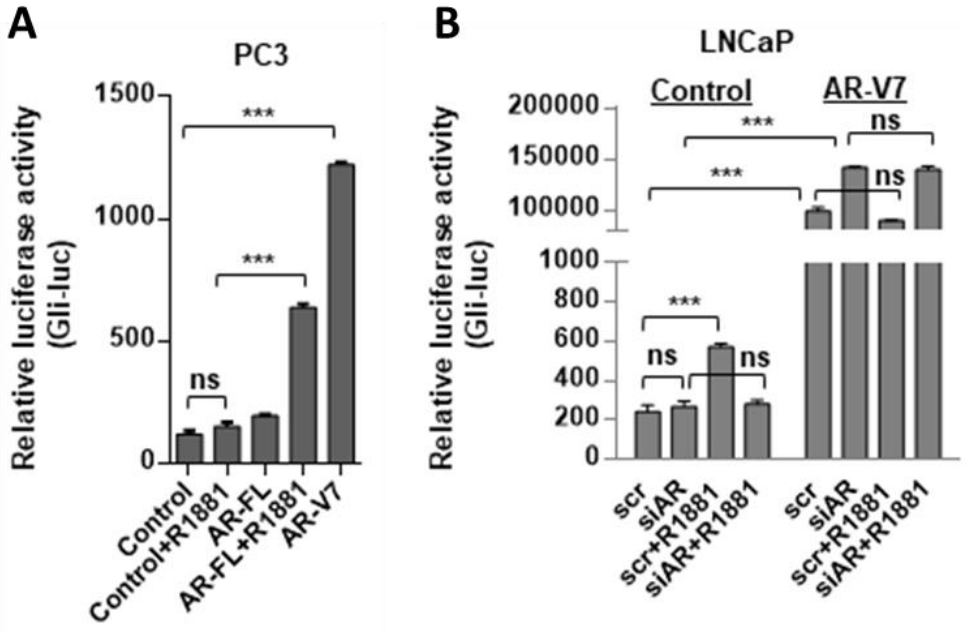

Figure 3.3 AR-V7 upregulates Gli-luciferase reporter activity in the presence or absence of androgen

Relative activity of a Gli-luciferase reporter assessed by luciferase assay in **(A)** PC3 cells transfected with empty vector control, AR-FL, or AR-V7, followed by treatment with vehicle or 1 nM R1881 for 48 hours and **(B)** LNCaP cells co-transfected with control (empty vector) or AR-V7 and scramble (scr) or siRNA targeting AR-FL (siAR), followed by treatment with vehicle or R1881 (1 nM) for 48 hours. Bars represent mean± SD, **P*<0.05; ****P*<0.001; ns, not significant.

3.1.4 Endogenous Gli target genes are upregulated by transcriptionally active AR

We also tested whether androgens increased expression of endogenous Gli target genes, Gli1 and Ptch1. LNCaP and LNCaP-AI cells cultured in charcoal-strip fetal bovine serum (CS-FBS) and then treated with vehicle or 1 nM R1881 showed increased mRNA expression of androgen-regulated genes PSA and KLK2, and Gli target genes Gli1 and Ptch1 **(Figure 3.4A)**. Significantly increased protein expression was also seen in these cells, along with similar upregulation of Gli target genes by androgen in LAPC4 cells **(Figure 3.4B)**.

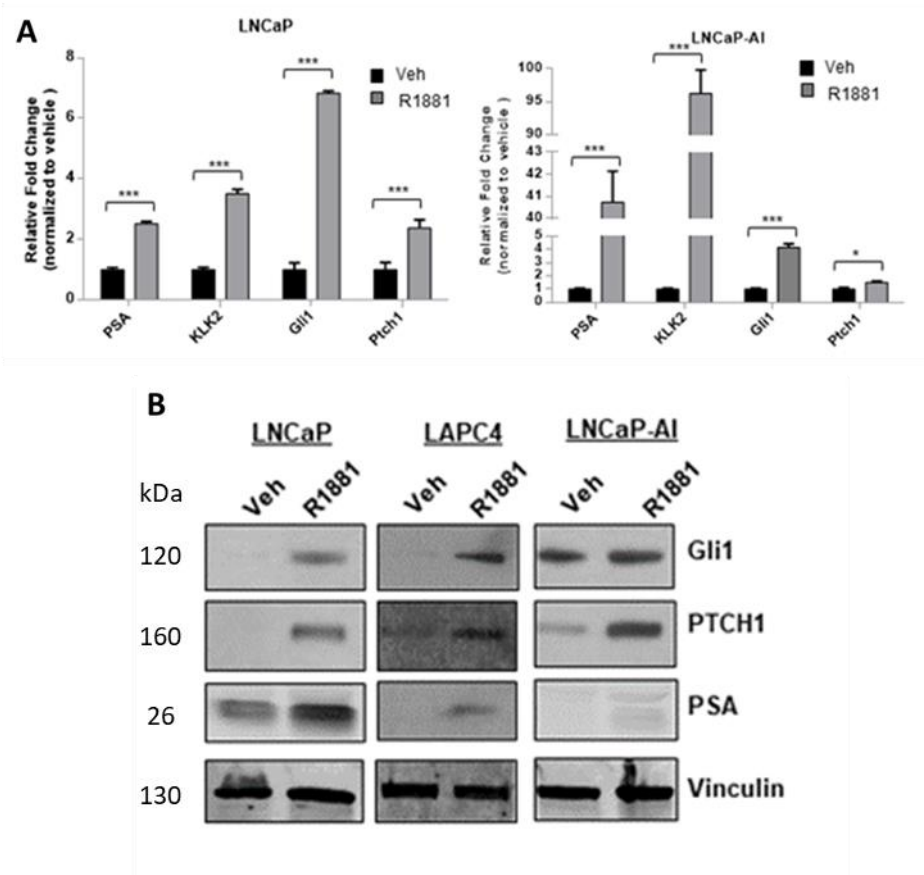

Figure 3.4 Expression of Gli target genes is upregulated in PCa cell lines

(A) Relative mRNA expression of AR target genes PSA and KLK2 and Gli target genes Gli1 and Ptch1 by quantitative real-time PCR (qRT-PCR) in LNCaP and LNCaP-AI cells cultured in charcoal-strip fetal bovine serum (CS-FBS), followed by treatment with vehicle or R1881 (1 nM) for 48 hours. 18s was used as a housekeeping gene and fold change was calculated using the $2^{(-\Delta\Delta CT)}$ method. Bars represent mean± SD, *P<0.05; ***P<0.001. **(B)** Protein expression of Gli1, Ptch1, PSA, and vinculin in LNCaP, LAPC4, and LNCaP-AI cells cultured in CS-FBS, followed by treatment with vehicle or R1881 (1 nM)

3.1.5 AR-driven Gli activity is higher than Smo-driven Gli activity

Since most prostate cancer cells are unresponsive to Smo antagonists, we used a mouse preosteoblast cell line, MC3T3-E1 cells, that is responsive to Smo agonists, to compare the effects of a chemical Smo agonist to exogenous overexpression of full-length or truncated AR (AR-V7) on Gli activity. While SAG treatment (200 nM) increased Gli-luciferase reporter activity by less than 1.5-fold, transient transfection of full-length (in the presence of 1 nM R1881) or truncated AR (without androgen) increased Gli reporter activity more than 10-fold higher than SAG treatment. **(Figure 3.5)**.

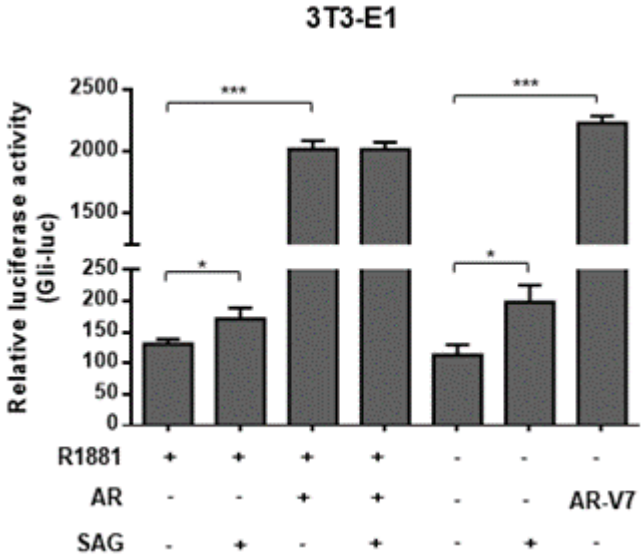

Figure 3.5 Transcriptionally-active AR enhances Gli-luciferase reporter activity in Smo-responsive mouse preosteoblast cells

Relative Gli-luciferase reporter activity assessed by luciferase assay in MC3T3-E1 cells serum starved in PBS for 24 hours, then transfected with control (empty vector), AR-FL, or AR-V7 in CS-FBS and treated with R1881 (1 nM) or SAG (200 nM) for 48 hours. Bars represent mean± SD, *P<0.05; ***P<0.001.

3.2 Characterization of the Gli landscape in PCa cells

In order to better understand the role of individual Glis in prostate cancer, we surveyed a number of prostate cancer cell lines and looked at the relative mRNA expression of Gli1, Gli2,

and Gli3 by quantitative real-time PCR. The predominant Gli gene expressed in all the PCa cell lines (except for LAPC4) is Gli3, oftentimes at >100 fold higher than Gli1 or Gli2 **(Figure 3.6)**.

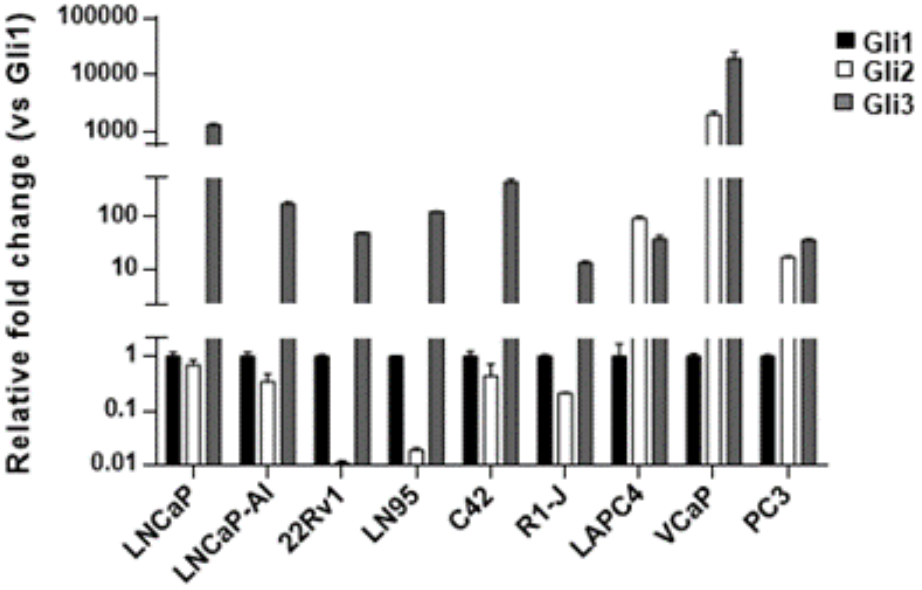

Figure 3.6 Gli3 is the predominant Gli expressed in PCa cell lines

Relative mRNA expression of Gli1, Gli2, and Gli3 in 9 prostate cancer cell lines, LNCaP, LNCaP-AI, 22Rv1, LN95, C42, R1-J, LAPC4, VCaP, and PC3. 18s was used as a housekeeping gene and fold change was calculated using the $2^{(-\Delta\Delta CT)}$ method. Data is shown as fold change relative to Gli1 expression levels; bars represent mean± SD.

3.3 Androgen promotes the interaction of AR with Gli3 in PCa cells

3.3.1 Gli3 content of AR immunoprecipitates is increased with androgen

To evaluate whether androgens affect the association between AR and Gli3 in vivo, we first determined how the presence of androgen (R1881) affected the amount of Gli3 protein that co-immunoprecipitated using an anti-AR antibody. Anti-AR co-immunoprecipitation was done on LNCaP cells, measuring endogenous Gli3-immunoprecipitation. Culture of LNCaP cells in CS-FBS followed by treatment with increasing amounts of androgen (R1881) showed an increase in active, full-length Gli3 content in AR immunoprecipitates, in a dose-dependent manner **(Figure 3.7)**. The Gli3 repressor (Gli3-R) did not bind to AR.

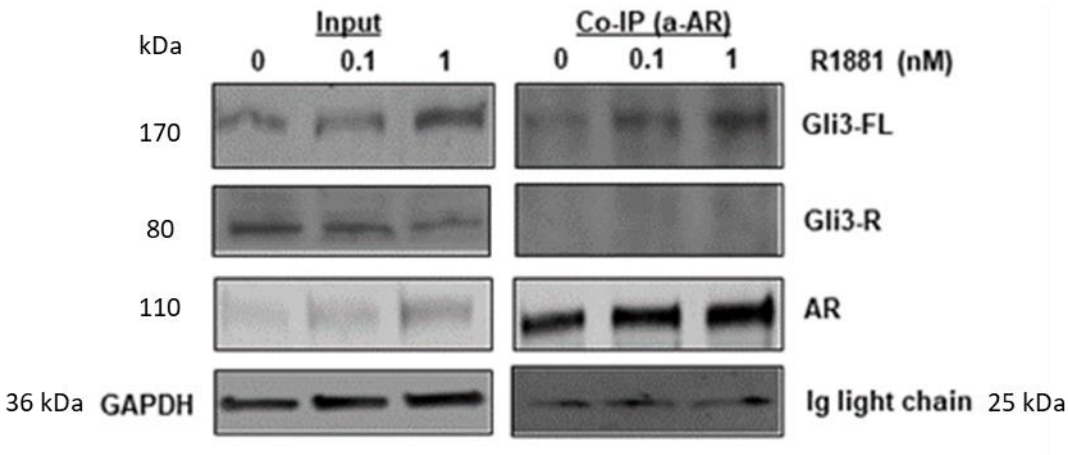

Figure 3.7 Androgen increases the Gli3 content in AR immunoprecipitates in a dose-dependent manner

Co-immunoprecipitation with an anti-AR antibody was performed on LNCaP cells cultured in CS-FBS and then treated with vehicle, 0.1 nM or 1 nM of R1881 for 48 hours. AR immunoprecipitates were blotted for Gli3 and AR; GAPDH was used as a loading control for input samples and IgG light chain was used as an internal control for immunoprecipitates.

3.3.2 Androgens increase the number of Gli3-AR complexes in situ

To verify that androgens promote Gli3-AR interactions, we also used proximity ligation assay. PLA is a procedure that identifies proteins in close contact *in situ*, to detect and relatively quantify Gli3-AR complexes in LNCaP cells treated with R1881 and/or enzalutamide. Addition of R1881 significantly increased the number of Gli3-AR complexes, which were mostly localized to the nucleus. Enzalutamide significantly reduced the number of complexes detected in the presence of R1881 **(Figure 3.8)**.

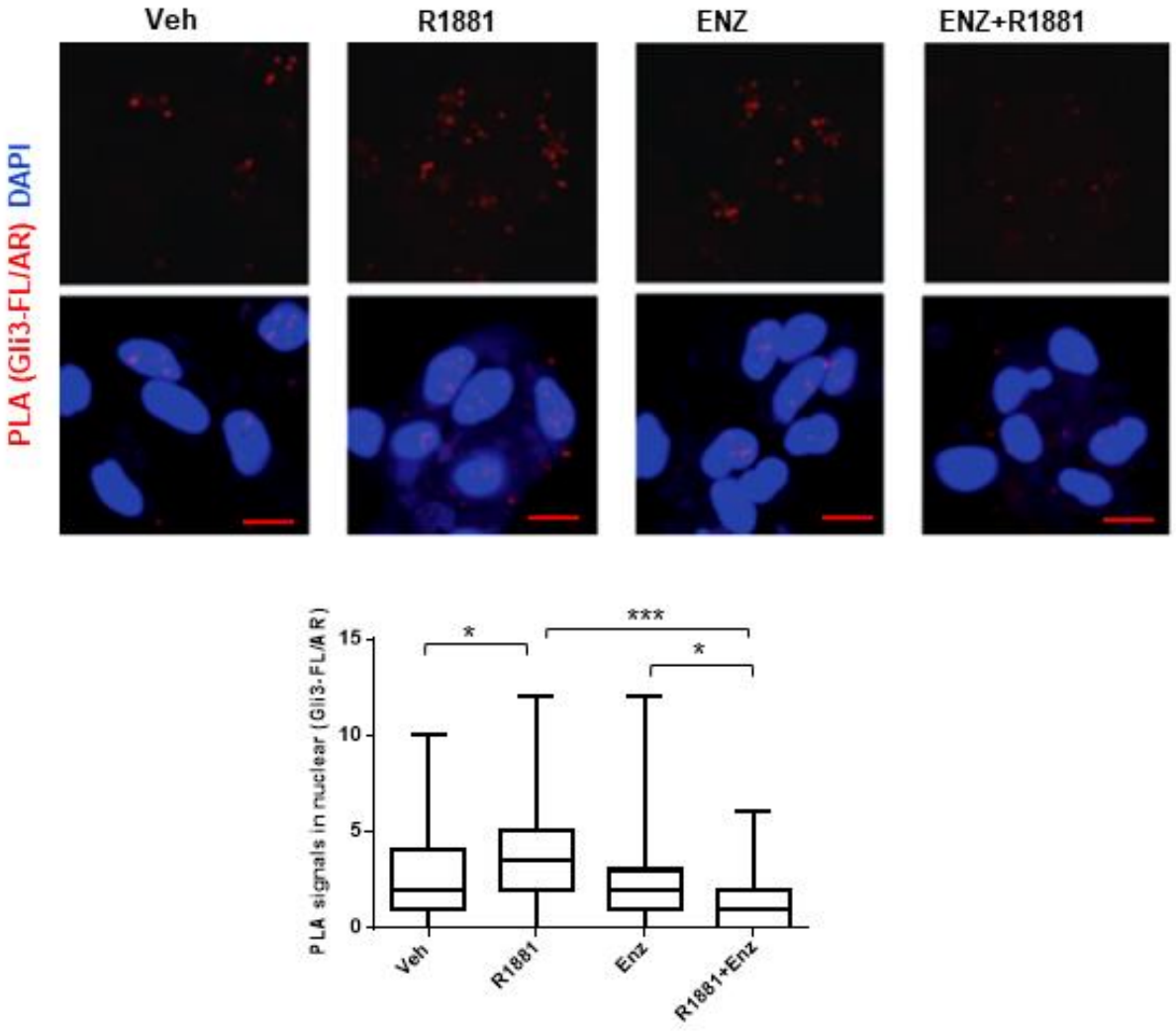

Figure 3.8 Androgen increases the number of Gli3-AR complexes detected by proximity ligation assay

LNCaP cells seeded on coverslips in CS-FBS then treated with vehicle (veh), 1 nM R1881, 10 µM Enzalutamide (Enz), or both for 48 hours. Representative images of each condition used for proximity ligation assay (top) and statistical analysis of quantification done using Duolink ImageTool software. Photomicrographs are shown at 40X, oil (scale bar = 20 µm). * $P<0.05$, *** $P<0.001$.

3.4 The AR binding site on Gli is involved in proteolytic processing of Gli proteins

3.4.1 The protein processing domain of Gli is located within the AR binding site

Previously, we showed that AR recognizes a C-terminal region of Gli2 that is adjacent to the Gli2 DNA binding domain. This region encompasses a polypeptide sequence that is highly homologous between Gli2 and Gli3. Although we found that PCa cell lines express Gli3 at much

higher levels than Gli2, we refined our characterization of this region on Gli2 as a means to better understand the nature of the interaction of AR with Gli proteins. The AR-binding site on Gli2 was further refined from a previously reported 270 amino acid region ($aa_{628-897}$) [82] to a 73 amino acid region ($aa_{764-836}$) using GST pull-down assays and site-specific mutagenesis. Deletion of the C-terminal end to aa836 or deletion of the N-terminal end to aa764 had no effect on binding to the Gli-binding domain of AR (AR-GBD), but deletion of the C-terminal back further to aa805 resulted in loss of binding **(Figure 3.9)**. This region is known as the protein processing domain (PPD) [86] and has previously been reported to be the target for site-specific phosphorylation and recognition by the E3 ligase, β-TrCP. [87]. Introduction of mutations within the arginine-, serine-rich repeats (SRRSS to SDDSS) at aa821, aa833, and aa864 in the $aa_{628-897}$ fragment of Gli2 resulted in loss of binding to AR-GBD **(Figure 3.9)**.

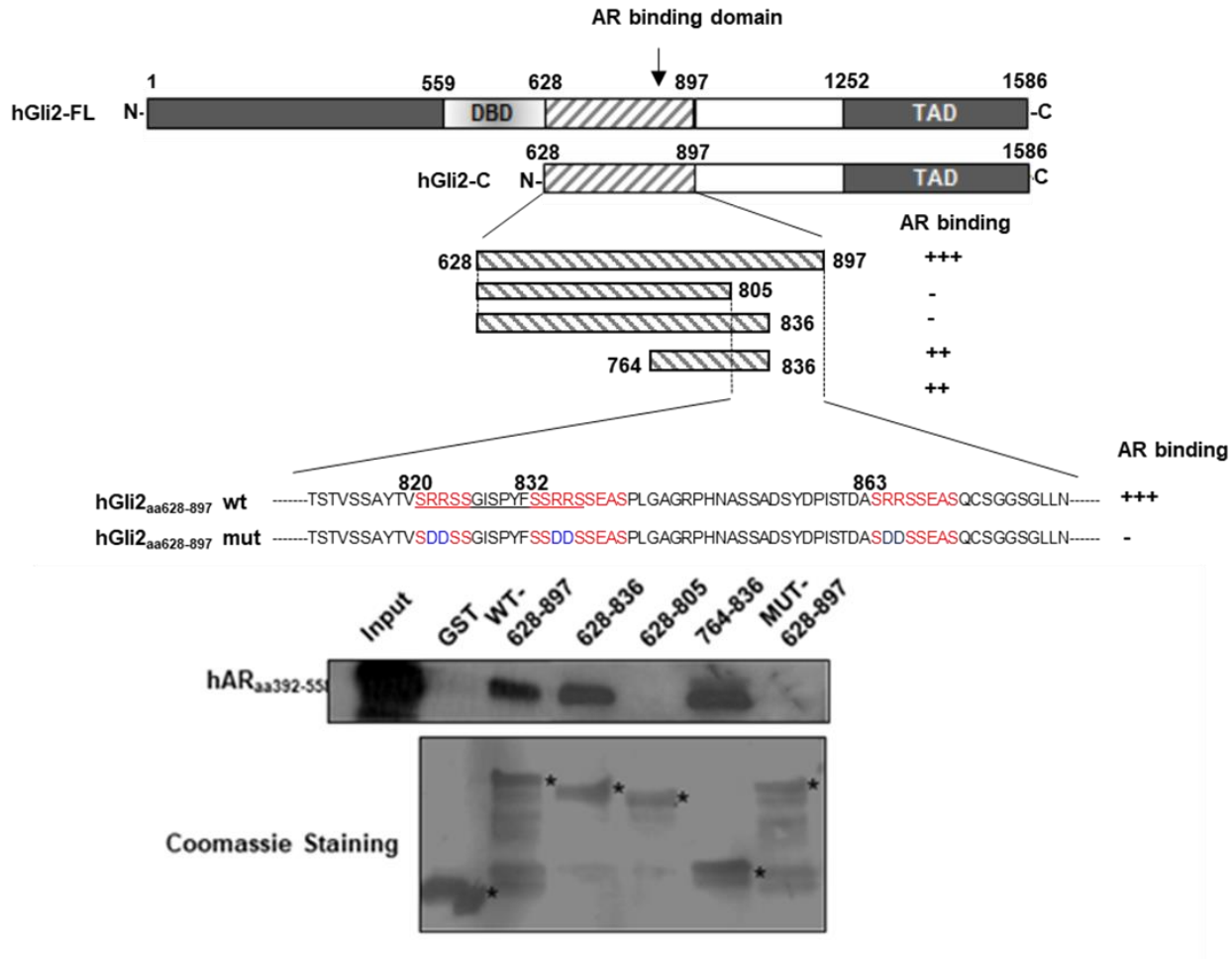

Figure 3.9 The AR binding site of Gli2 encompasses the protein processing domain of Gli

(Upper), Schematic diagram of Gli2 protein domains and various sub-fragments (below) tested for binding to AR-GBD (hARaa$_{392-558}$) in GST-pulldowns with binding outcomes indicated on the right. (Middle) Partial sequence of Gli2aa$_{805-897}$ showing phosphorylation site (-S-R-R-S- repeats [red]) and below, sequence of mutated Gli2aa$_{805-897}$, as was described. (Bottom) Retention of his-tagged AR-GBD polypeptide (Upper) on recombinant GST-tagged Gl2 polypeptides affixed to glutathione beads confirmed the minimum AR binding domain at Gli2aa$_{764-836}$. Eluates were resolved on SDS-PAGE and western blots were probed for AR-GBD using an his antibody as described. (Lower) Gli2 recombinant proteins were visualized under Commassie staining (* indicates expected size) after SDS-PAGE.

3.4.2 AR competes with β-TrCP for binding to Gli

We first used an in vitro assay to test whether AR and β-TrCP compete for the same binding site on Gli2. A Gli2 aa$_{764-836}$ polypeptide was affixed to glutathione beads, then incubated with the purified N-terminus of AR (aa$_{1-558}$) in the presence of increasing concentration of recombinant β-TrCP. After washing, the beads were eluted with SDS

containing loading buffer and the eluted proteins were resolved on SDS-PAGE. The results **(Figure 3.10A)** show that AR binding was diminished by the presence of β-TrCP, showing that AR competes with β-TrCP for binding to Gli2. We also observed this in vivo where transfection of increasing amounts of β-TrCP expression vector displaced AR from Gli3 immunoprecipitates **(Figure 3.10B)**.

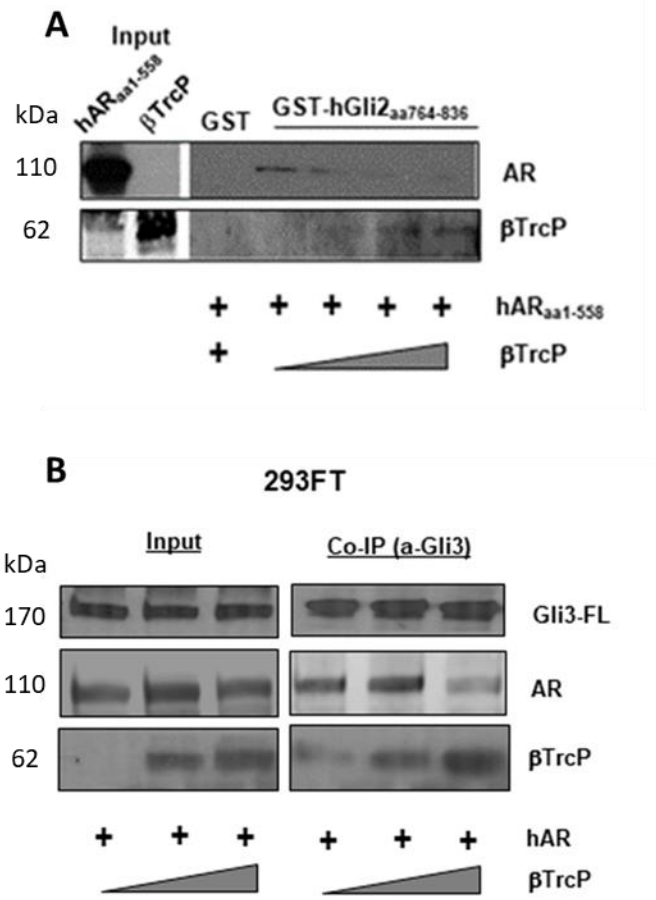

Figure 3.10 β-TrCP competes for binding of Gli with the N-terminus of AR

(A) Coomassie stained GST pull-down of AR N-terminus and Gli2 $aa_{764-836}$ in the presence of varying concentrations of β-TrCP (ratio of β-TrCP:AR 0:1, 1:4, 1:2, and 1:1). GST beads alone were used as a negative control **(B)** Co-immunoprecipitation of AR-overexpressing 293-FT cells cultured in DMEM + 10% FBS with varying ratios of β-TrCP:AR (ratio of β-TrCP:AR 0:1, 1:1, 2:1)

3.5 Androgens alter the processing of Gli3

3.5.1 Proteolytic processing of Gli3 is altered by androgens in a dose-dependent manner

We looked at the effect of androgens on the processing of Gli3 proteins to determine a mechanism for the effect on Gli transcription. LNCaP cells grown in androgen-depleted medium (CS-FBS) have a low ratio of active Gli3-FL to Gli3-R (repressor) and addition of increasing doses of androgen (R1881) showed a progressive increase in the ratio of Gli3-FL:Gli3-R (**Figure 3.11A)** in treated cells. This shows that androgens can stabilize high molecular weight Gli3 and suppress Gli3-FL processing to the truncated repressor form. Correspondingly, increased Gli-luciferase activity was also seen with increasing doses of androgen **(Figure 3.11B)**.

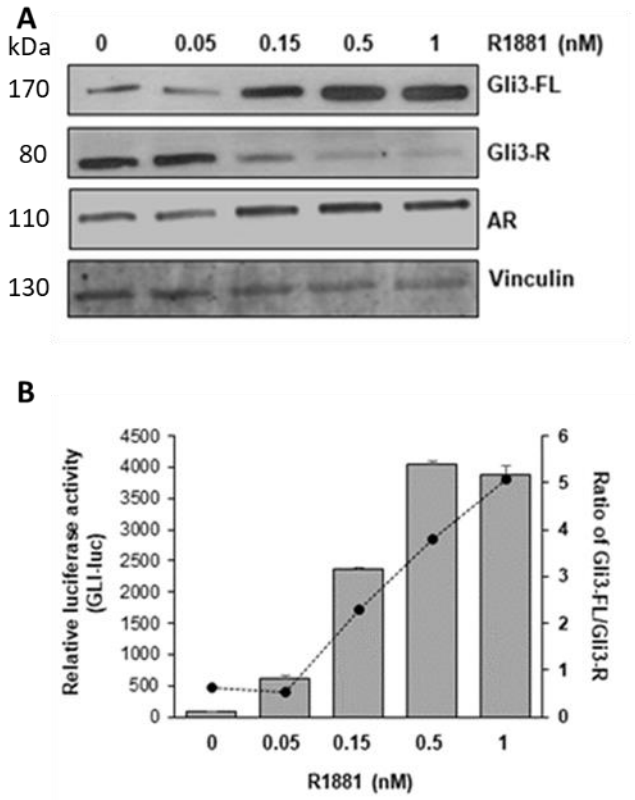

Figure 3.11 Increasing doses of androgen alter the ratio of Gli3-FL:Gli3-R and induce Gli-luciferase activity

(A) Western blots showing protein expression of Gli3 (-FL and –R forms), AR, and vinculin and **(B)** relative Gli-luciferase activity of LNCaP cells cultured in androgen-depleted medium, then treated with increasing doses of R1881 as shown for 48 hours.

3.5.2 The effect of AR-FL knockdown on Gli3 processing

We next tested whether knockdown of AR by siRNA affected the ability of androgens to stabilize high molecular weight Gli3. As we previously observed in LNCaP and now in LNCaP derivatives (LNCaP-AI and LN95), the ratio of Gli3-FL to Gli3-R was increased with androgen even in the presence of a non-targeting siRNA (scr vs. scr + R1881), while knocking down AR-FL with siRNA blocked stabilization of high molecular weight Gli3-FL in both the absence and presence of androgen **(Figure 3.12)**.

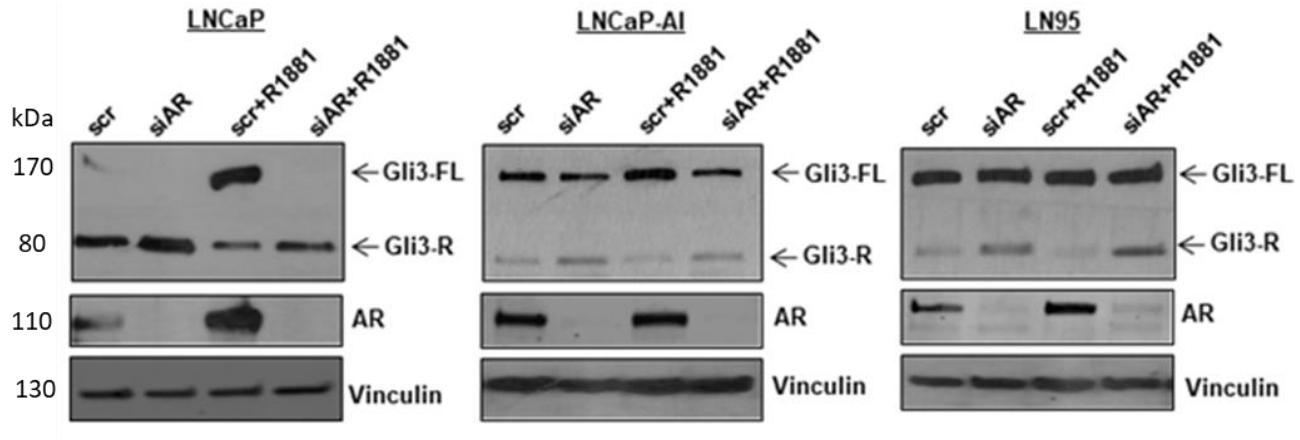

Figure 3.12 siRNA knockdown of AR-FL alters the ratio of Gli3-FL:Gli3-R

Protein expression of Gli3, AR, and vinculin in LNCaP, LNCaP-AI and LN95 cells cultured in CS-FBS, transfected with scr or siRNA targeting AR-FL, followed by treatment with vehicle or R1881 (1 nM) for 48 hours.

3.6 Decoy peptides from the Gli2 C-terminal domain compete with Gli3 for AR binding and inhibit Hh signaling/Gli activity in PCa cells

3.6.1 A Gli2 decoy peptide competes with Gli3 for binding to AR

We tested whether a 270 aa peptide derived from the C-terminal AR binding domain of Gli2 would serve as decoy to interfere with Gli3-AR interaction. Exogenous overexpression of this decoy peptide in LN95 cells followed by AR co-immunoprecipitation showed displacement of Gli3 from AR immunoprecipitates **(Figure 3.13a)**. *In situ* proximity ligation assay shows that

overexpression of the Gli2 decoy peptide (Gli-DP) also decreases the number of Gli3-AR complexes in LN-AI cells overexpressing dox-inducible myc-tagged Gli-DP **(Figure 3.13b)**.

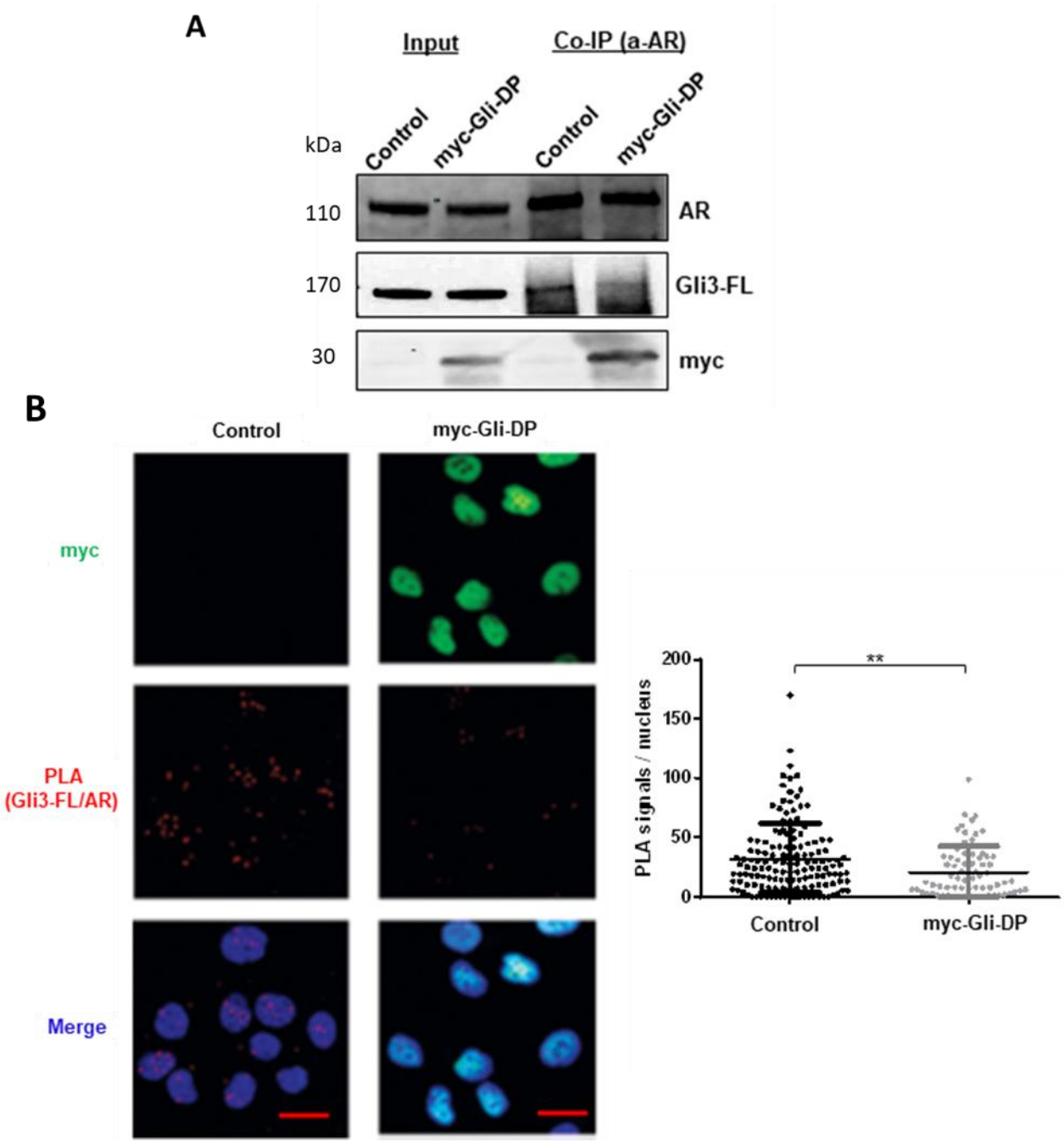

Figure 3.13 A Gli2 decoy peptide competes with Gli3 for binding to AR *in vivo* and *in situ*

(A) AR co-immunoprecipitation of LN95 cells overexpressing a 270 aa Gli2 decoy peptide **(B)** Representative proximity ligation assay (PLA) images and graphical representation of PLA quantification in LN-AI cells overexpressing dox-inducible myc-Gli-DP or control vector. Photomicrographs are shown at 40X, oil (scale bar = 20 µm).

3.6.2 Gli transcriptional activity is suppressed by a Gli2 decoy peptide

We also looked at the effect of the Gli2 decoy peptide on Gli transcriptional activity. LNCaP cells were co-transfected with the Gli-luciferase reporter plasmid and the Gli2 decoy peptide, then treated with vehicle or 1 nM R1881. Overexpression of the Gli2 decoy peptide reduced luciferase reporter expression **(Figure 3.14a)**. Additionally, we looked at Gli1 and Ptch1 mRNA by qRT-PCR in LNCaP and LNCaP-AI cells overexpressing the Gli2 decoy peptide. The decoy peptide suppressed expression of both Gli1 and Ptch1 mRNA **(Figure 3.14b)**.

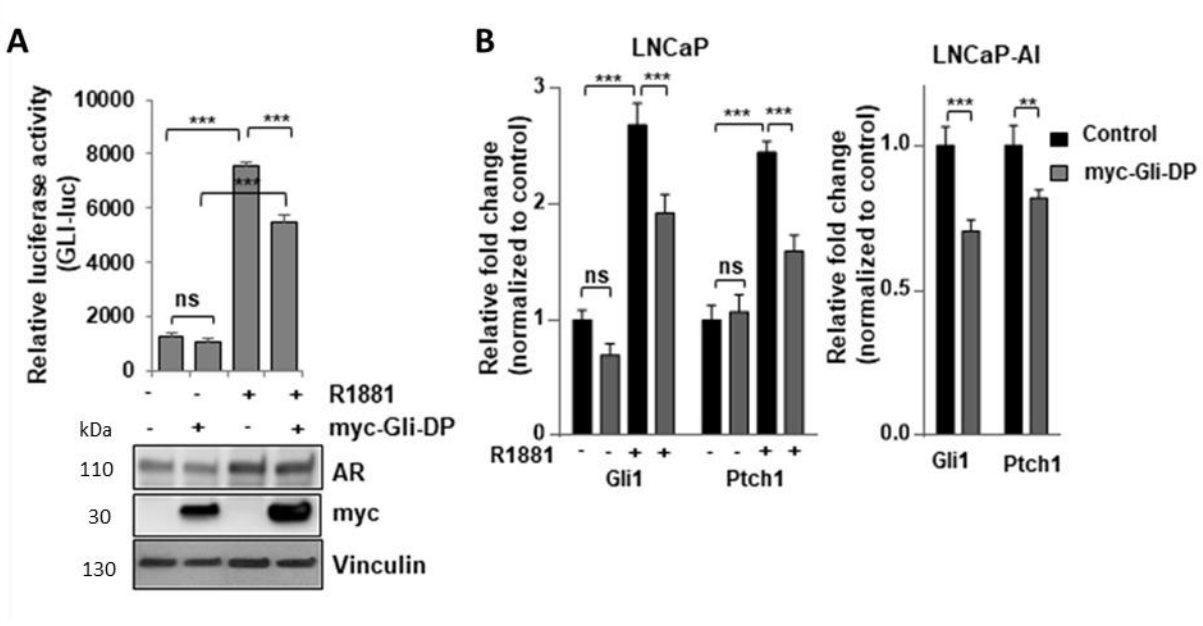

Figure 3.14 Gli-luciferase reporter activity and Gli target genes are suppressed by a Gli2 decoy peptide
(A) Relative Gli-luciferase reporter activity assessed by luciferase assay for LNCaP cells transiently transfected with an empty vector control or a 270 aa Gli2 decoy peptide (myc-Gli-DP) then treated with vehicle or 1 nM R1881 for 48 hours **(B)** Gli1 and Ptch1 mRNA assessed by qRT-PCR for LNCaP and LNCaP-AI cells overexpressing a dox-inducible control vector (empty) or a Gli2 decoy peptide (myc-Gli-DP). 18s was used as a housekeeping gene and fold change was calculated using the $2^{(-\Delta\Delta CT)}$ method. Bars represent mean± SD, *P<0.05; ***P<0.001; ns, not significant.

3.7 Transcriptionally active estrogen receptor and glucocorticoid receptor also upregulate Gli transcriptional activity.

Finally, we tested whether other steroid receptors, specifically estrogen receptor alpha and glucocorticoid receptor, might have similar Gli-activating properties. Transient transfection

of 293FT cells with an estrogen receptor alpha (ER) or glucocorticoid receptor (GR) expression vector followed by treatment with vehicle or their respective steroid (10 nM or 50 nM estradiol, or 5 nM or 10 nM dexamethasone) showed upregulation of Gli-luciferase reporter activity, similar to the effect of transcriptionally active AR **(Figure 3.15)**.

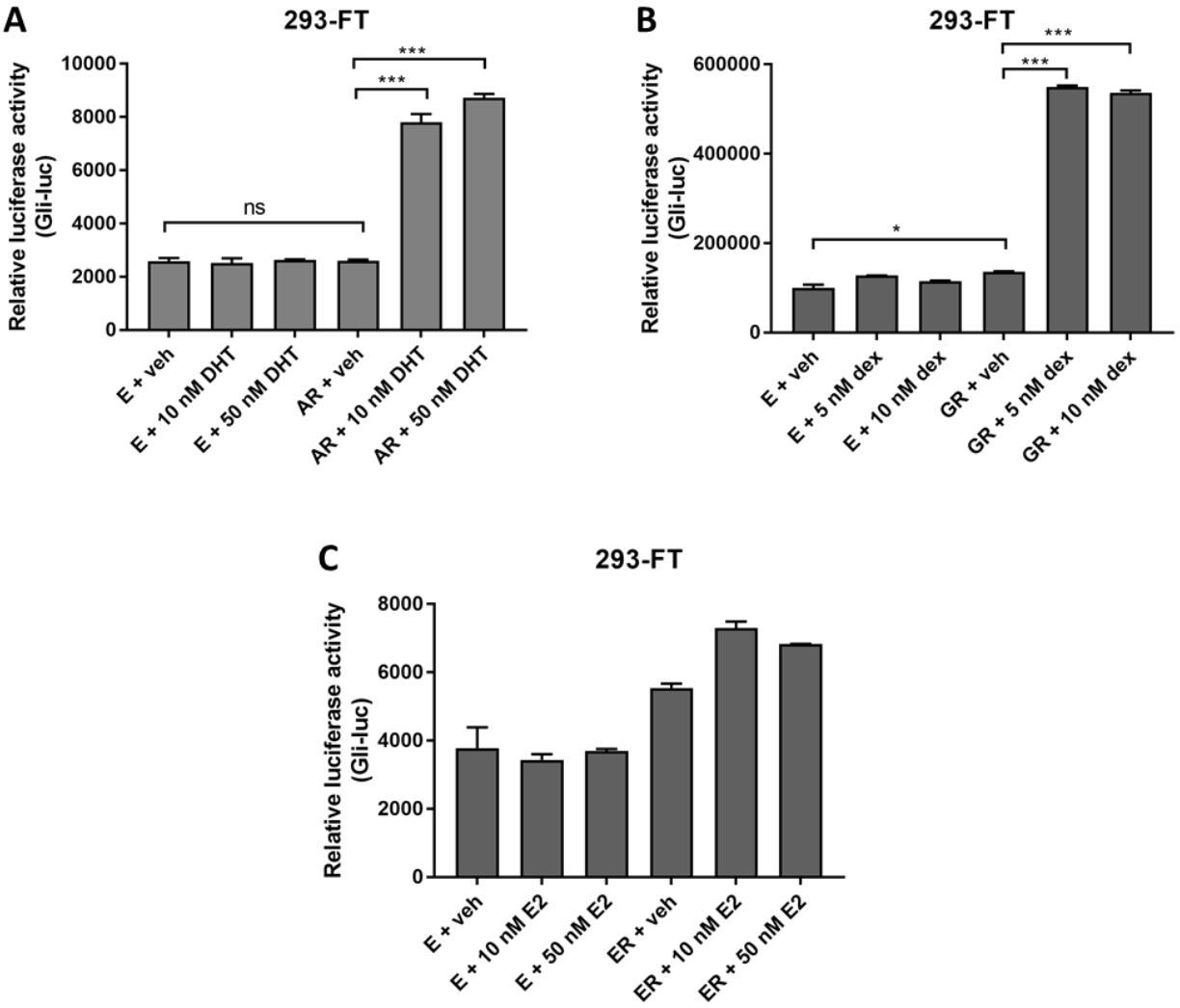

Figure 3.15 Transcriptionally active ER and GR induce Gli-luciferase reporter activity

Relative Gli-luciferase reporter activity of 293-FTcells transiently transfected with empty vector (E) or AR **(A)**, GR **(B)**, or ER **(C)**, followed by treatment with vehicle or steroid (10 nM or 50 nM DHT, 10 nM or 50 nM estradiol, or 5 nM or 10 nM dexamethasone). Bars represent mean± SD, **P*<0.05; ****P*<0.001; ns, not significant.

Chapter 4: Discussion

Prostate cancer, when caught early, has a good prognosis and can often be cured by surgery or radiation. Advanced (metastatic) prostate cancer, however, is a problematic disease that rapidly develops resistance to the hormone therapies used to control it as well as chemotherapeutics that are used when hormone therapies fail. Standard androgen deprivation therapies are no longer able to slow disease progression, as cells develop mechanisms to overcome the lack of androgens, although most CRPCs remain androgen receptor-dependent. Researchers' attention has therefore turned to targeted treatments to combat these mechanisms, and the Hh pathway has long been a pathway of interest in prostate cancer. This is due to the abundance of evidence for overexpression of hedgehog ligands, overexpression of Gli proteins, and evidence that Gli suppression can reduce the growth of PCa cells.

Whereas unrestrained Hh signaling is sufficient for oncogenesis in the brain and skin, increasing evidence indicates that Hh cooperates with the androgen signaling system of PCa cells to effect the development of therapeutic resistance. For one, androgens suppress the expression of Hh ligands in AR+ PCa cells. Androgen deprivation causes significant upregulation of Hh ligand expression [79] which can then signal, through paracrine stimulation of benign stromal cells in the tumour microenvironment, to produce steroids needed for tumour growth [28]. This activity is driven by canonical Hh signaling and it can be suppressed with a Smo antagonist. In striking contrast, Hh activity in PCa cells appears to be mostly refractory to Smo antagonists. Instead, there appears to be a mutually-supportive, bi-directional interaction between the AR and Gli proteins in PCa cells that contributes to a resistant phenotype.

Previously, we described the evidence showing that Gli proteins are co-activators of the androgen receptor in PCa cells. Knockdown of Gli reduced the ability of AR to induce androgen-

responsive genes. We have found that the interplay between the AR and Hh pathways also includes a unique bi-directional relationship between the transcription factors that define both of these pathways, AR and Gli proteins. AR is a critical regulator of androgen signaling, with roles not only in prostate development, but also in prostate diseases, serving as an important therapeutic target for PCa treatments. The Hh pathway is best known as an embryonic developmental pathway, but Gli proteins also appear to play a role in prostate development. Investigations have shown and continue to show that the Hh pathway and Gli transcription factors play an important role in advanced prostate cancer, contributing to cell growth [67, 76]. Aberrant Hh signaling in prostate cancer has been reported in various studies, but little to no evidence suggests that the process is Smo-driven. A lack of primary cilia in cancer cells supports the idea that Hh pathway activation is non-canonical. In BCC and medulloblastoma, mutations in Smo and other upstream regulators of the Hh pathway have been found, but no similar mutations have been reported in prostate cancer. The data presented in this thesis supports the idea that binding of transcriptionally activated AR to Gli proteins results in co-activation of Glis, causing non-canonical Hh pathway activation, potentially driving tumour growth.

We previously showed that Gli proteins bind directly to a region of AR containing the core sequence of Tau5, co-activating both full-length and truncated forms of the receptor [82], and here we showed that the reverse is also true. This hypothesis initially arose in the wake of AR ChIP data that showed an accumulation of AR at a Gli-response element on the Ptch1 gene in Gli2-overexpressing LNCaP cells. The availability of online datasets allowed us to search in the Cistrome project and find AR ChIP data showing accumulation of AR at the Gli-response element of another Gli target gene (Gli1), supporting the hypothesis and prompting further research into the co-activation of Gli by binding of AR.

Using a Gli-driven luciferase reporter, we found that in AR+ prostate cancer cell lines containing full-length AR, treatment with androgens upregulated expression of the Gli-luciferase reporter. This could be blocked by the addition of enzalutamide, an AR antagonist, or by siRNA knockdown of full-length AR, indicating that the effects of androgen on Gli-reporter activity required the presence of transcriptionally active AR. Addition of androgen and/or enzalutamide had no effect on the R1-d567 cell line, as it expresses only a truncated AR variant, AR-e567 which is insensitive to androgens. We found that overexpression of a constitutively active, truncated AR, AR-V7, also upregulated Gli transcription, an effect that was further enhanced (in both the presence and absence of androgens) by knockdown of full-length AR. Our work with a Smo-responsive mouse fibroblast cell line indicates that AR may be a much more robust activator of Gli activity compared to Smo.

Aside from the Gli-luciferase reporter that was used, we also looked at the mRNA and protein expression levels of endogenous Gli target genes, Gli1 and Ptch1, in our PCa cell lines. Androgen supplementation upregulated PSA and KLK2, as expected. But androgens also upregulated Gli1 and Ptch1 mRNA and protein.

Our gene expression studies established that Gli3 is the predominant homologue of Gli expressed in PCa cells, so we focused on characterizing the effect of androgen on Gli3 proteins. Co-immunoprecipitation of LNCaP cell lysates and immunoblotting for AR and Gli3 showed that androgens increased the Gli3 content of AR immunoprecipitates, suggesting that transcriptionally active AR selectively complexes with Gli3. This was confirmed in our proximity ligation assays that showed that in situ Gli-AR complexes were increased by androgen stimulation.

Previously, the AR binding domain on Gli2 was reported to be a 270 peptide region between amino acids 628-897. We further refined it to a 73 amino acid region of Gli2 764-836 by deleting portions of the C- and N-terminus to find the minimal AR-GBD binding peptide. This region encompasses the "protein processing domain" (PPD) of Gli2 and Gli3 that contains a series of short serine-, arginine-rich repeats. By mutating the Arg residues to Asp, we found that we lost binding to the AR-GBD. The serines in these PPD repeats are phosphorylated by PKA, GSK3 and CK1, and this recruits the ubiquitin ligase, β-TrCP. Ubiquitination of Gli by β-TrCP leads to a site-specifc degradation of Gli3 that removes the C-terminal transactivation domain resulting in a functional repressor Gli3 protein (Gli3R) [40]. As such, we tested whether AR binding to Gli3 might compete with β-TrCP for binding to Gli3 and also whether AR binding affects the proteolytic processing to Gli3R. Indeed, we found through in vitro studies that β-TrCP competes with AR for binding to Gli3. We also showed that AR binding to Gli3 blocks its degradation to Gli3R and stabilizes the high molecular weight form of Gli3.

Since we have already established the AR binding domain on Gli2, we used the 270 aa polypeptide as a decoy peptide (Gli-DP) to engage AR in PCa cells and displace Gli3-FL. This effect was confirmed by a loss of Gli3 in AR co-immunoprecipitates and a decreased number of Gli3-AR complexes in PCa cells overexpressing the Gli2 decoy peptide. Consequences of Gli-DP mediated disruption of Gli3-AR interactions was a drop in Gli-luciferase reporter activity and decreased expression of endogenous Gli target genes. Disruption of the Gli3-AR interaction may prove to be an effective therapeutic target for metastatic PCa, as Hh/Gli is known to regulate genes involved in cell proliferation and epithelial-mesenchymal transition (EMT).

The effect of transcriptionally active AR on Gli2 is an issue that has yet to be addressed, and thus far has proven difficult due to lower expression levels of Gli2 relative to Gli3 and

technical difficulties with antibodies against Gli2. This may be overcome in the future with the use of cell lines with higher expression of Gli2 such as VCaP and LAPC4.

The novel link between transcriptionally active AR and Gli activity adds to the growing body of research into the role that the Hh pathway plays in PCa growth and progression. In androgen-dependent CRPC, both AR signaling and the Hh pathway play important roles in tumour growth and impeding cross-talk between the two pathways by interfering with the interaction of AR and Gli may prove to be efficacious in preventing disease progression.

In summary, our study shows a novel role for transcriptionally active AR in non-canonical Hh pathway activation by binding directly to and co-activating the Gli transcription factors. We show that AR and β-TrcP compete for binding to Gli proteins. Competitive binding by AR provides a possible mechanism by which Gli evades proteosomal degradation and activates Gli target genes, contributing to cell growth and disease progression. A Gli2 decoy peptide can be used to displace full-length Gli3 and disrupt the Gli3-AR interaction in PCa cells.

In addition to our data on activation of Gli by AR, preliminary data on the effect of other steroid receptors on Gli proteins has shown that estrogen receptor (ER) and glucocorticoid receptor (GR) may function in a similar manner as AR. Experiments with overexpression of GR and ER in 293-FT cells followed by treatment with steroid shows upregulation of Gli-luciferase activity, but more work is required to establish a relationship between these signaling pathways and Gli activation.

Chapter 5: Study limitations and future directions

This work established that AR recognizes and binds to the Gli PPD which is a site of phosphorylation and ubiquitination that regulates a proteasome specific proteolysis. While this study has shown that AR competes with β-TrcP for binding to the Gli3 PPD, phosphorylation and ubiquitination studies were not done to show that the binding of AR affects these processes. Follow-up studies using phospho-specific antibodies for western blot or ELISA, as well as western blots combining anti-ubiquitin and target protein antibodies could help us determine this. One of the limitations of this project thus far has been a lack of *in vivo* data, as all experiments were done *in vitro*, in cell lines or using synthesized recombinant proteins. Additional investigation needs to be done into whether non-canonical Hedgehog signaling via androgen receptor binding to Gli occurs *in vivo* in mouse tumour xenograft models, as well as in human pathological samples to determine the relevance of this project to the biology of PCa. Furthermore, this study has focused on Gli3, as the predominant Gli homologue expressed in PCa cells, but the role of Gli2 needs to be studied as well. Gli2 is also expressed in PCa cells and may play a compensatory role when Gli3 is inhibited. Finally, the ability of AR to activate reporter expression from a Gli-dependent promoter suggests that AR binding to DNA is not needed for Gli activation. Further work could be done to determine whether mutated ARs that lack DNA binding are able to bind to, stabilize, and transcriptionally activate Gli3.

In the future, development of a small molecular weight inhibitor might prove to be a useful therapeutic tool for CRPC patients. The Gli2 decoy peptide used in this study will need further study to determine whether it affects cell growth and tumour progression both *in vitro* and *in vivo*. Further refinement and/or crystallization and structural studies of the AR and Gli binding sites will be required for development of a small inhibitor.

Although this study has limitations and further investigation is needed, in the end, this work suggests that steroid receptors might have two functions: activation of a gene expression program through enhanced transcription of steroid-regulated genes and co-activation of Hedgehog signaling.

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