ROLE OF RECEPTOR TYROSINE KINASE LIKE ORPHAN RECEPTOR 2 IN TREATMENT-RESISTANT PROSTATE CANCER

by

Chihwan (Paul) Ahn

B.Sc., The University of British Columbia, 2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2020

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Role of receptor tyrosine kinase like orphan receptor 2 in treatment-resistant prostate cancer

submitted by Chihwan Ahn in partial fulfilment of the requirements for

the degree of Master of Science

in Experimental Medicine

Examining Committee:

Dr. Amina Zoubeidi, Professor, Department of Urologic Sciences, UBC

Supervisor

Dr. Wilfred Jefferies, Professor, Department of Microbiology and Immunology, UBC

Supervisory Committee Member

Dr. Vincent Duronio, Professor, Department of Medicine, UBC

Supervisory Committee Member

Dr. Caigan Du, Associate Professor, Department of Urologic Sciences, UBC

Additional Examiner

Abstract

Potent androgen receptor pathway inhibitors such as Enzalutamide (ENZ) and Abiraterone (Abi) have become the gold standard for patients with castration resistant prostate cancer (CRPC). However, treatment resistance is inevitable and all patients eventually become insensitive to these treatments. To investigate the molecular mechanism of treatment resistance in prostate cancer, our laboratory has engineered ENZ-resistant (ENZ^R) CRPC cell lines, which mirror clinical observations, through serial passaging of LNCaP xenografts under ENZ. Using our RNA sequencing data, we found Receptor tyrosine kinase like orphan receptor 2 (ROR2) as one of the most upregulated receptor tyrosine kinases (RTKs) in both our in vitro cell lines and patients. The oncogenic roles of ROR2 have been elucidated in various cancers, including prostate cancer. However, the role of ROR2 in context of treatment resistance in prostate cancer is still unknown. For the first time, we generated a novel ROR2 gene signature to provide insights on its role in treatment-resistant prostate cancer using RNA sequencing data of in vitro models and patients. We successfully validated the legitimacy of ROR2 gene signature in various models. Correlation studies revealed that ROR2 activity may be ligand-independent. Further examination of ROR2 gene signature revealed that ROR2 upregulates CD274 (known as programmed death-ligand 1; PD-L1) in treatment-resistant setting. Various computational studies and in vitro experiments supported and validated the novel ROR2-CD274 axis. Together, our data reveal a novel discovery of ROR2-CD274 axis in treatment-resistant prostate cancer.

Lay Summary

Prostate cancer is the most common cancer among Canadian men. Currently, there are number of treatments available for patients with advanced prostate cancer. However, majority of patients with advanced disease will develop resistance over time and will fall to the disease. This study examines changes that occur when the cancer becomes resistant to current treatments. This study could hold the key to understanding the process of treatment resistance in prostate cancer and guide new treatment development.

Preface

This dissertation is an original product of the author, Chihwan (Paul) Ahn. The data presented in this thesis are based on the work I carried out during the completion of my M.Sc. program. Dr. Amina Zoubeidi was the principal investigator in this study.

Table of Contents

List of Tables

List of Figures

Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisor, Dr. Amina Zoubeidi. Thank you for giving me the opportunity to be part of your successful team and believing in me throughout my studies. Your courage and work ethic were inspirational and your unconditional support guided me to success. This work was not possible without you, Amina.

Secondly, I would like to thank my supervisory committees, Dr. Vincent Duronio and Dr. Wilfred Jefferies for their knowledge and guidance. Your supports gave me confidence to my research and allowed me to finish the project. Thank you, Vince and Wilf.

I would like to also thank all the members of Vancouver Prostate Centre (VPC) for making the journey enjoyable. Cheers to you all.

In addition, I would like to show my appreciation to my friends outside of VPC. The support you guys gave me got me through the toughest times and this would not have been possible without you. Thank you.

Lastly, I would like to thank my family Jimmy, Hye Ran, Kwang Ok, for your endless love. Thank you for providing a loving environment for me.

Cheers to everyone around me!

Dedication

To my family and friends

1. Introduction

1.1. Prostate Cancer

1.1.1. The prostate

The prostate gland, more accurately labelled as the prostate muscle, is the largest accessory gland in the male reproductive system composed of unstriped muscular, fibrous, elastic, connective, glandular, nerve, vascular, lymphatic and a small amount of striped muscular tissue [1]. It is located inferior to the urinary bladder, posterior to the lower portion of the symphysis pubis and anterior to the rectum. Known to be "walnut-shaped", the prostate gland surrounds the proximal urethra [2].

Structurally, the prostate gland is composed of an inner layer (consists of smooth muscle) encapsulated in collagen-containing fibroelastic outer layer. The outer surface of the prostate gland is homogenous in nature and lacks exterior lobation. The interior of the prostate gland can be subdivided into five lobes: an anterior, a posterior, a medial and two lateral lobes. These lobes are composed of dense stroma, blood and lymphatic vessels, nerves, 30-50 branched tubule-alveolar or saccular glands, and 16-32 excretory ducts [3]. These lobes become more apparent in benign prostatic hyperplasia.

Histological classification of prostate gland results in the division of the organ into three major zones (peripheral zone (PZ), central zone (CZ), transition zone (TZ)) and anterior-fibromuscular stroma (AFS). These zones have different embryological origins, appearances, biological functions and structures. The peripheral zone is the largest zone in the prostate gland, comprising approximately ~70% of the glandular tissue [4]. The peripheral zone contains relatively simple branching of the duct system, loose stroma and evenly distributed acinar elements. The peripheral zone is the portion of the gland that can be felt during the digital rectal exam. The central zone, located at the base on the prostate between PZ and TZ, is a cone-shaped zone that surrounds the ejaculatory duct and narrows at the verumontanum (a longitudinal mucosal fold that marks the point where the ejaculatory ducts enter the urethra) [2]. It accounts for approximately ~25% of the glandular tissue and consists of an elaborate duct system, dense stroma and lobular acinar arrangements [3]. The transition zone accounts for ~5% of the glandular tissue and consists of two small lobes that surround the proximal urethra. This is the zone that enlarges during benign prostatic hyperplasia [2]. Lastly, the anterior fibromuscular stroma forms the exterior surface of anterior side of prostate gland. Unlike the other three zones, AFS is composed of fibrous and smooth muscle instead of glandular tissues. Majority of the prostate cancer (~70%) originates in the peripheral

zone, followed by the transition zone (~25%). Figure **1.1.** illustrates histologically classified zones of prostate gland.

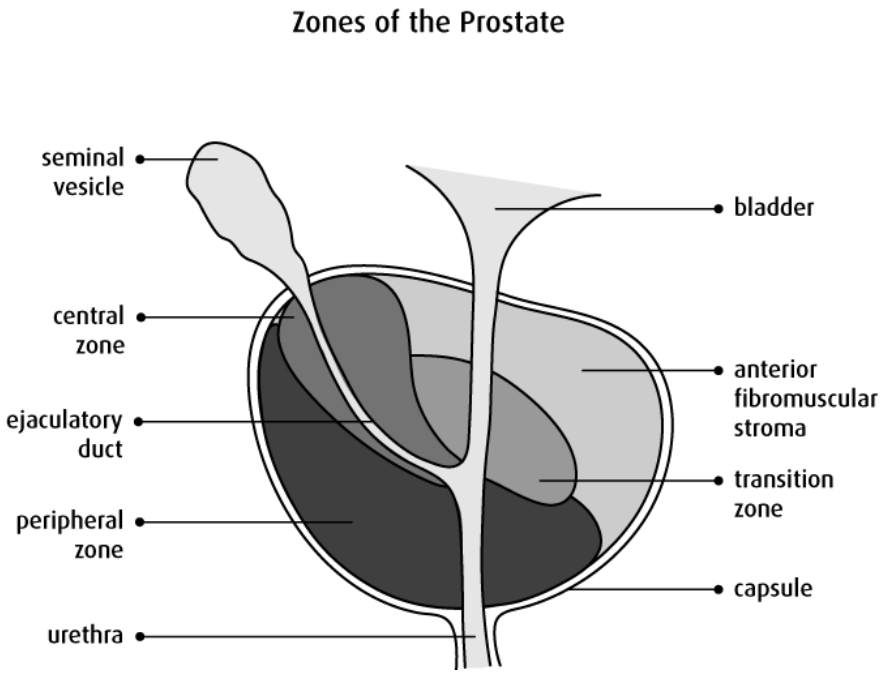


Figure 1. 1. Sagittal view of the prostate gland. Going from the posterior to anterior, the prostate gland is classified into peripheral zone, central zone, transition zone, and anterior fibromuscular stroma. In respect to the size, the peripheral zone makes up the majority of the gland.

Functionally, the prostate gland carries out various functions in which the main function is to secrete the prostatic fluid, an alkaline fluid that is a component of semen. The prostatic fluid improves the sperm viability by reducing the acidity and enhances sperm motility by providing albumin to the seminal plasma that stimulates the motility [5]. It also contains high level of zinc acting as an antibacterial agent in seminal fluid, and prostatic acid phosphatase which converts phosphorylcholine to choline which acts as nutrients for sperm cells [5]. Other functions include control of the urine output from the bladder and in the transmission of the semen during ejaculation; producing small molecules and enzymes (such as coagulase and fibrinolysin) that facilitate fertility and rapidly metabolizing testosterone into more potent form of androgen called dihydrotestotsterone (DHT) [3, 5].

1.1.2. Epidemiology of prostate cancer

Prostate cancer is the second most frequently diagnosed cancer (13.5% of total incidence in men) and fifth leading cause of death (with the mortality rate of 6.7%) amongst men in the world [6]. Rates of incidence and mortality vary based on the geographical area with western countries and some European countries having higher rates than Asian countries. In Canada, prostate cancer is more prevalent with it being the most common cancer in men and is the 3rd leading cause of death. Over the years, a number of different factors have been identified as contributors to the risk of prostate cancer, including dietary, life-style and genetic factors. For example, ethnicity and race are known contributors to the risk of prostate cancer, with highest risk of prostate cancer being present in African-American men [7]. Consumption of food high in soy and vegetables such as tomatoes, garlic, and cruciferous vegetables are known to reduce the susceptibility to prostate cancer while diet rich in red meat and dairy products are known to be associated with higher risk [8, 9]. Genes that acts as a significant risk factor for the development of prostate cancer are risk alleles in hereditary prostate cancer (HPC) and inheritance of these risk alleles can increase the incidence as well as accelerate the onset time (<60 years old). One example of these HPC risk alleles is *HPC1* which encodes for enzyme ribonuclease L (RNASEL). RNASEL is involved in the innate immune response as well as interferon-mediated signaling [10]. Functional data supporting the role of RNASEL in HPC is evidently shown in cases of prostate cancer patients that harbor germline truncating mutation (E265X) in *HPC1*, resulting in the loss of heterozygosity of wild type allele [10]. Among different factors associated with the risk of prostate cancer, age is the most significant risk factor; the prevalence of prostate cancer drastically goes up from 23.4% in 50-59 year old group to 45.5% in 70-81 year old group [11]. Furthermore, older men are more likely to be diagnosed with higher-risk prostate cancer and lower survival rate [10].

1.1.3. Detection and diagnosis of prostate cancer

Prostate Specific Antigen (PSA) screening and digital rectal exam (DRE) are the 'gold standards' for early detection of prostate cancer. PSA screening test measures the PSA level in the blood sample drawn from a vein in your arm while DRE is a physical examination where a doctor puts a gloved, lubricated finger into the rectum of the patient to examine for abnormalities such as enlargement of the prostate gland or growths. Although implantation of PSA screening significantly improved early detection of prostate cancer and lead to decrease in prostate cancer-related deaths, the screening is not cost-effective and false positive rate is high. Furthermore, advanced high-grade prostate cancers can exhibit low serum PSA levels and may be missed by the current guidelines for PSA screening test [12]. Therefore, there has been tremendous efforts being made to improve the detection method. For example, additional biomarkers

(such as TMPRSS2:ERG fusion and PCA3) have been proposed to be included in the screening test [13]. Despite its controversy over the clinical efficacy, PSA screening test combined with DRE acts as the initial detection methods for prostate cancer.

If abnormalities are detected in DRE or PSA test, additional tests can be further used for diagnosis. These tests include transrectal ultrasound (TRUS), tissue biopsy, MRI, blood chemistry tests, bone scan, and CT scan. TRUS utilizes an ultrasound probe in the rectum to create an image of the prostate. In prostate biopsy, a thin, hollow needle or probe is used to collect small tissue sample which is further analyzed in the pathology laboratory for diagnosis. Blood chemistry tests measure organ/tissue-specific biomarkers (such as blood urea nitrogen for kidney function or alkaline phosphatase for bone) to help with diagnosing the stage of prostate cancer. Bone scan and CT scan can also be used to determine whether metastasis have occurred in various other body parts. Combinations of different diagnostic tests can verify whether an individual has prostate cancer as well as characterizing the disease.

1.1.4. Gleason scoring system

Prostate cancer can be divided into different grades (from low to high) based on their morphology and histological features. The grading scheme for prostate cancer is developed initially by pathologist Donald F. Gleason in 1974 and became known as 'Gleason Scoring' system. The Gleason scoring system is based on the architectural features (not cellular characteristics) and the overall score is determined by the sum of two most relevant growth patterns. The conventional (or classic) Gleason scoring system consists total of five growth patterns with Gleason pattern of 1 representing the best differentiated and most favourable prognosis while Gleason pattern of 5 having severely disarrayed glandular structure and least favour and generally a poorer prognosis. Specifically, Gleason pattern 1 is characterized by nodular lesion composed of compact, well-differentiated, moderately sized glands. In the current understanding, Gleason pattern 1 is considered as adenosis or atypical adenomatous hyperplasia (AAH), not bona fide prostate adenocarcinoma due to its rarity. Gleason pattern 2 consists of neoplastic glands of varying sizes with increased stroma and irregularity in the nodular periphery. Gleason pattern 3, which is the most common pattern in patients with prostate cancer, comprised of distinct neoplastic glands with variable size and shape that are infiltrating into stroma in between normal glands. Gleason pattern 4 consists of fused glands, irregular glandular (or cribriform) pattern and lumen. Gleason pattern 5 is characterized by the presence of necrosis and individual tumor cells without any glandular formation [14]. Despite changes in diagnostic and prognostic tests in prostate cancer, Gleason scoring system remains one of most powerful predictors in prostate cancer. However, the current Gleason scoring system has undergone

various modifications from its original one in the effort to improve the efficiency and accuracy of the system. Based on the 2005 International Society of Urologic Pathology (ISUP) Consensus, changes were made in the attempt to address controversial issues originated from the conventional system. Examples of the revision include: 1) Gleason patterns 1 and 2 (scores of 2-5) are no longer assigned on needle core biopsy due to its poor reproducibility and correlation with radical prostatectomy grade 2) rearrangement of previously diagnosed Gleason pattern 2 adenocarcinomas into Gleason grade 3 3) inclusion of glomeruloid morphology as part of Gleason pattern 4 [15]. Modification to the classical Gleason scoring system resulted in a shift toward higher Gleason scoring; it was found that there was an increase of Gleason score 7-10 diagnoses from 59% to 72% [16]. Furthermore, modified Gleason scoring system brought improved overall reproducibility, raising the interobserver reproducibility up to 80% [14]. **Figure 1.2.** shows typical patterns of the modified Gleason grading system and corresponding scores.

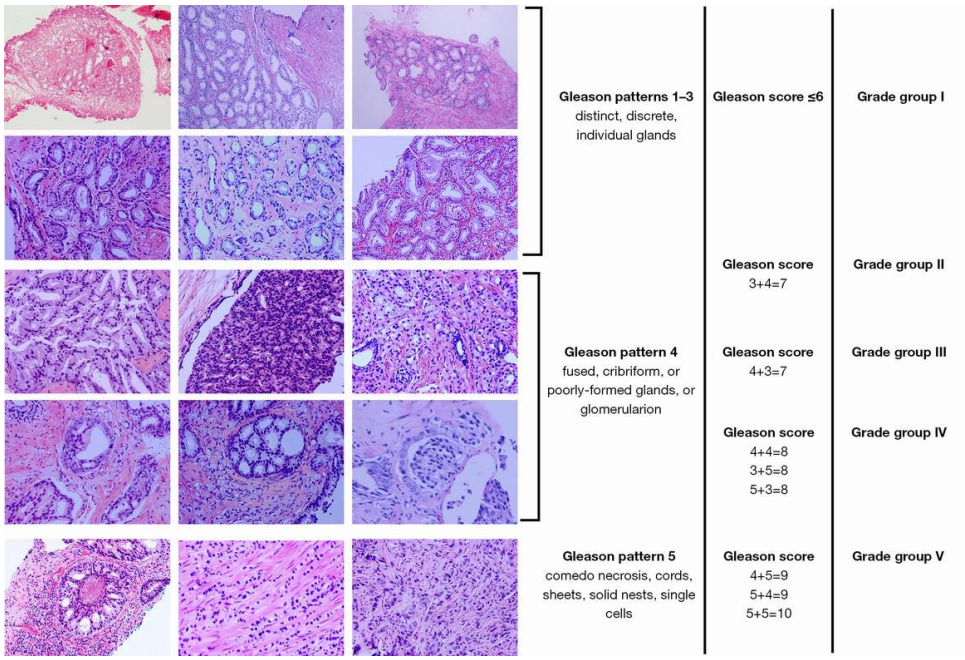


Figure 1. 2. Modified Gleason Scoring System. Histological patterns (left) are specific to each Gleason pattern (middle) and subsequently to Gleason score (right), which is the sum of two most relevant patterns. Based on the Gleason patterns and scores, prostate cancer can be classified into Grade groups, ranging from group I to V. This figure was extracted from Chen et al. (The evolving Gleason grading system, CJCR, 2016) with the permission granted by Elsevier.

1.1.5. Prostate cancer development and biology

Prostate cancer, by definition, is the malignancy of the prostate gland. More specifically, it is a type of carcinoma that involves the accumulation of malignant epithelial cells in the prostate gland, but other non-epithelial cell types can play a role in initiation and progression of the disease. Prostate cancer can be sub-divided into series of clinical states and transitions from each state are accompanied by histological and molecular changes. The normal prostate is considered to be quiescent, but this can change in benign prostatic hyperplasia (BPH) which results in hyper-plastic prostate epithelium [17]. BPH is not a precursor for prostate cancer [18]. In contrast to BPH, high-grade prostatic intraepithelial neoplasia (PIN), is considered to be a precursor for prostate cancer. High-grade PIN and prostate cancer have extensive overlap in genetic and molecular markers and PIN acts as a clinical intermediate state between benign epithelium and malignant carcinoma. Furthermore, evidence that high-grade PIN serves as a premalignant stage is reinforced by the fact that it's onset predates prostate cancer by few years and it's incidence and severity increases with old age [19]. Histologically, high-grade PIN is described to have 4 major patterns, including tufting, micropapillary, cribriform, and flat [20]. PIN is described by loss of basal cell layer integrity (basal cells are still present) and accumulation of epithelial cells with nuclear enlargement [21]. The transition from high-grade PIN to prostate cancer (the most common type of prostate malignancy is adenocarcinoma) is characterized by multiple histological changes in invasive epithelium. These changes include loss of basal cell layer, excessive branching morphogenesis, and cytologic atypia.

The initiation of prostate cancer primarily depends on early genetic and epigenetic event that occur during transition from the normal prostate. Epigenetic changes leading to increased methylation and subsequent silencing of critical genes are amongst earliest events during initiation [22]. Some of these critical genes that are silenced through hyper-methylation include *GSTP1, APC, RABB2,* and *MDR1* [23]. Furthermore, overexpression of SPINK1 and ETS-family genes such as *ETV4* and *ETV5*, have been described to facilitate in the initiation process [24, 25]. Genome-wide profiling of gene expressions of prostate cancers and PINs reveal that the prostate carcinogenesis from PIN involves changes in genes involved in cell adhesion or motility such as *POV1, CDKN2C, APOD, EPHA4, FASN, ITGB2, LAMB2,* and *TIMP1* [26]. These are just some of the examples of complex array of genetic changes that occur during the initiation of prostate cancer. These genetic changes provide insight into the magnitude and complexity of the carcinogenesis of prostate cancer and in partial explains the late onset of prostate cancer. It also suggests that there are several different subtypes of prostate cancer driven by different molecular mechanisms.

1.1.6. Androgen receptor (AR) signaling pathway in prostate cancer

Prostate cancer is a hormone-dependent disease and in the center of its pathogenesis lies the androgen receptor (AR) pathway. Approximately ~90% of prostate cancer depends on androgen at its initial diagnosis [27]. Androgen receptor plays a crucial role in the normal development and maintenance of the prostate gland and it remains important in prostate cancer development and progression. The hormonal dependence of normal prostate was first described by Scottish surgeon John Hunter in 1786, but it was not until 1941, by Huggins and Hodges, that the significance of androgen pathway in prostate cancer development and progression was elucidated [28]. They reported that orchiectomy (removal of the testes) and estrogen injection lead to regression of prostate cancer (as measured by serum phosphatase) while androgen injection resulted in the opposite [29]. AR is a ligand-dependent transcription factor that belongs to a steroid hormone group of nuclear receptors along with estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR). AR binds to endogenous androgens testosterone and more potent 5α-dihydrotestosterone (DHT) and the binding of AR to its native ligands initiates the androgen pathway. Androgen pathway begins when circulating testosterone that are functionally active (~3% of total) enters the cytoplasm of prostate cells and is converted into DHT by 5α-reductase enzyme. DHT binds to ligand-binding domain of AR with high affinity and promotes dissociation of heat-shock proteins from AR. This leads to nuclear translocation of AR. In the nucleus, AR dimerizes and binds to androgen response element (ARE) in the promoter region of target genes and recruit members of transcription machinery (such as TATA-box binding protein (TBP) and co-regulators, leading to transcription of target genes such as *KLK3* and *TMPRSS2* [30]. Expression of various target genes promote survival and proliferation of prostate cancer cells while preventing apoptosis. Although the AR expression in present in primary prostate cancer and can be detected in the advanced diseases, immunohistochemical analysis reveal that AR expression is heterogeneous. AR heterogeneity does not generally correlate with how patients respond to disease, although there are evidences of AR expression positively correlating with lower Gleason scores. **Figure 1.3.** illustrates the role of AR in molecular pathogenesis of prostate cancer.

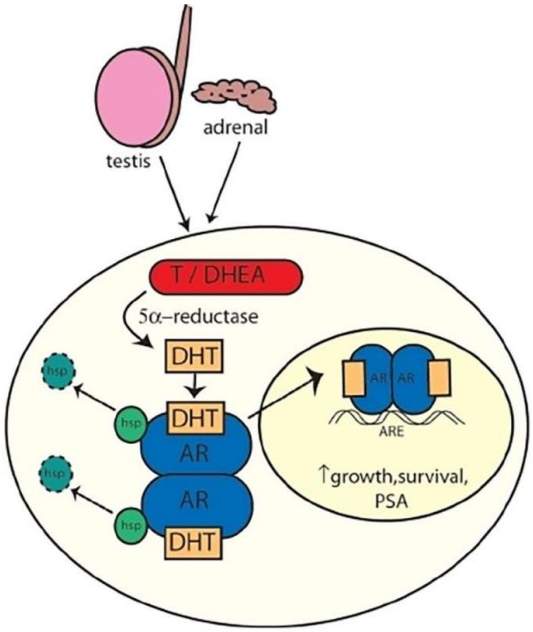


Figure 1. 3. Androgen Receptor pathway in prostate cancer. Testosterone (produced by either testis or adrenal gland) in the blood enter the cytoplasm of prostate cancer cells, in which get converted into DHT by 5α-reductase. AR binds to DHT, dimerizes, and enters the nucleus where it binds to ARE of various genes and transcribes genes vital for cell growth and survival. This figure was provided by National Cancer Institute (https://www.cancer.gov/news-events/cancer-currents-blog/2016/prostate-ror-gamma-inhibitor).

1.1.7. Progression of prostate cancer and treatments

Majority of prostate cancer at diagnosis is an adenocarcinoma that is described as an 'androgen-driven' disease that depends on AR signaling for tumor survival and growth. Therefore, targeting components of androgen receptor pathway as well as depleting androgens have been well-rationalized mainstay for treatments in prostate cancer. In majority of cases, localized prostate cancer (defined by no identifiable lymph nodes and distant metastases) are treated with one of three option: 1) expectant management 2) surgery 3) radiation therapy. Expectant management consists of monitoring the progression of the disease through active surveillance (ie. prostate biopsies, regular PSA testing, physical examinations) and watchful waiting without any clinical treatments [31]. Expectant management is suitable for patients with none to low significant localized diseases. Surgery and radiation therapy remain as preferred choices when it comes to more significant, higher risk localized disease. The surgery is known as radical prostatectomy, a surgical removal of a complete prostate gland [32]. For patients with advanced, metastatic diseases or high-grade localized disease that cannot be treated with the options above or recurring diseases,

androgen deprivation therapy (ADT) is the 'gold standard' treatment regime. Its primary objective is to significantly reduce the level of androgens [33]. The conventional form of ADT in use worldwide is bilateral orchiectomy (surgical castration by removing testicles). While the conventional surgical castration is cost effective, it has fallen out of favour for its psychological burden and has been replaced by medical castration. Medical castration involves administration of gonadotropin-releasing hormone agonists (GnRH agonists) to block testosterone production. GnRH agonists suppress the production of luteinizing hormone by homologous desensitization (suppression of gonadotropin secretion by desensitizing GnRH receptor to stimulants) which in turn, suppress the production of androgens in the testes [34, 35]. Two commonly used GnRH agonists are Leuprolide and Goserelin. Majority of patients with advanced disease initially respond well to ADT, showing partial or complete regression [36]. However, all patients that are treated with ADT will eventually progress. ADT can provide a selective advantage for prostate cancer cells that possess molecular capacity to survive and grow in androgen-deprived condition while providing selective pressure for other cancer cells to acquire resistance. Currently, various resistance mechanisms have been discovered including AR overexpression, AR mutations, modification of co-regulators, AR hypersensitivity, AR variants, androgen-independent AR activation, intra-tumoural androgen production. These castration/ADT-resistant cancer cells will grow and repopulate the tumour, forming castration-resistant prostate cancer (CRPC). The definition of CRPC can be either clinical (development of metastatic disease, progression of pre-existing disease) or biochemical (three consecutive rises in PSA levels above nadir) in the presence of castrate levels of circulating testosterone [37]. Patients with CRPC will eventually succumb to the disease.

For CRPC, new therapeutic approach is required. In the recent years (since 2010), significant amount of efforts has gone into developing novel therapeutics for castration-resistant prostate cancer [38]. One of major therapeutic approaches used for CRPC patients is novel hormone therapies. Androgen signaling remains central to CRPC and identification of second-generation anti-androgens have allowed the development of novel drugs of clinical significance. Enzalutamide (FDA approved in 2012) and Abiraterone (FDA approved in 2011) are two examples of novel antiandrogens that clinics have benefited from. Enzalutamide interferes with androgen-receptor signaling by directly binding to AR and inhibiting 1) androgen binding to AR 2) nuclear translocation 3) DNA binding of AR/androgen complex [39]. Abiraterone (Abiraterone Acetate) is cytochrome p450 17A1 (CYP17) inhibitor that prevents androgen production [40]. Other therapeutic approaches include chemotherapy (ie. Docetaxel, Cabaxitaxel, Mitoxantrone), radium-223, and immunotherapy (Sipuleucel-T). However, despite these efforts to treat CRPC, treatment resistance is inevitable and disease will progress. Majority of CRPC (~75%) will become

resistant to AR reactivation and hypersensitivity and clinical and molecular features resemble the one of resistance to ADT [36]. In the other patients (~25%), the disease relapses in an AR-indifferent manner, characterized by having features of neuroendocrine small-cell carcinoma, and thus known as neuroendocrine prostate cancer (NEPC). This is quite remarkable since de novo development of NEPC is extremely rare (1-2%) and this shows the capacity of prostate cancer to exhibit cellular plasticity. Molecularly, NEPC is characterized by loss of *TP53* or *RB1*, upregulation of *EZH2,* and gain of *MYCN* or *AUKRA* . Clinically, NEPC is characterized by low serum PSA and AR negativity [36]. Patients with clinical relapse are limited to palliative care and non-specific chemotherapetuics and will eventually succumb to the disease. **Figure 1.4.** illustrates the clinical timeline of prostate cancer disease progression and corresponding treatments at each diseae state.

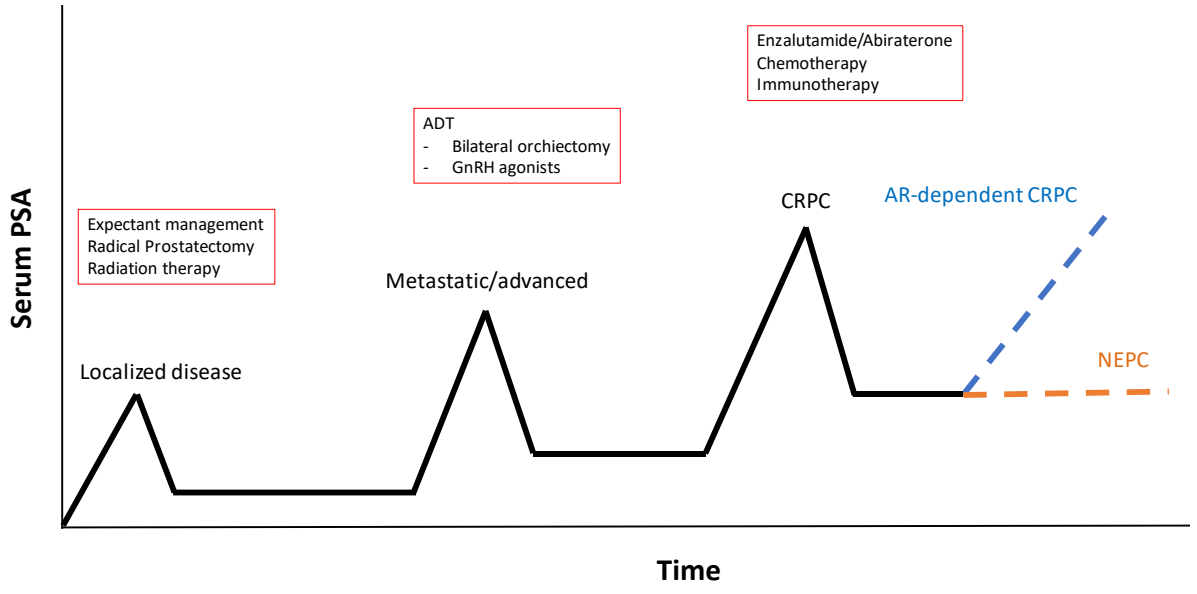


Figure 1. 4. Clinical timeline of prostate cancer and corresponding treatments. Localized disease is usually treated by active surveillence, radical prostatectomy, or radiation therapy. Once the disease becomes advanced or metastatic, ADT is utilized. If the disease progress despite being on ADT or metastatic relapses occur, CRPC is clinically diagnosed and treated with second-generation anti-androgens (Enzalutamide and Abiraterone), chemotherapy, or immunotherapy. However, patients will develop resistance to these therapeutic interventions through either AR-dependent (~75%) or AR-independent NEPC (~25%). This figure was adapted from Davies et al. (Nature Reviews Urology, 2018).

1.2. Receptor Tyrosine Kinase Like Orphan Receptor 2 (ROR2)

1.2.1. Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) are a subclass of cell-surface receptors with intracellular tyrosine-kinase activity. They play an important role in regulating diverse cellular functions such as proliferation, migration, metabolism, differentiation and survival in normal cells as well as having crucial implications in oncogenesis [41]. Humans have 58 known RTKS to date, which belong to 20 subfamilies. **Figure 1.6.** illustrates human RTK subfamilies and receptors that belong in each subfamily. Despite their sub-classifications, all RTKs have similar molecular architecture; the 'consensus' structure of RTK consists of 1) extracellular ligand-binding domains 2) single transmembrane helix 3) cytoplasmic tyrosine kinase domain and carboxyl terminal region 4) juxtamembrane regulatory regions. Furthermore, mechanism of activation, key intracellular signaling pathways as well as the overall topology are highly conserved in evolutionary point of view [42]. In general, the mechanism of receptor activation involves ligand binding (ie. growth factors) to corresponding RTKs, which induces receptor dimerization. It is important to note that certain subsets of RTKs can form oligomers independent of activating ligands (ie. insulin and IGF1 receptors) or form larger oligomers upon binding rather than dimers (ie. Tie2 and Eph receptors) [42]. Furthermore, EGFR family members EGFR and ErbB2 are known to transduce signals to their ligands EGF or NRG through hetero-oligomerization [43-45]. Whether the 'inactive' receptors are monomeric or oligomeric, ligand binding is required in both cases to activate the receptors through stabilization. In general, ligand-induced dimerization can occur through range of mechanisms based on varying degree of involvement of ligand in dimer interface (all, a portion, or none); more specifically, two extremes are "ligand mediated" (two receptors make no direct contact and ligands make up all the dimer interface) and "receptor mediated" (no direct contribution of ligands to dimer interface), and the rest involve a combination of ligand mediated and receptor mediated components [42]. Although majority of human RTKs are likely to use one of described mechanisms above, unique variations in the mechanism of receptor activation are observed in certain RTKs. For example, two human discoidin domain receptors DDR1/2 are activated by collagen fibres rather than soluble growth factors while MuSK is activated through indirect interaction with its ligand and requires accessory molecule Lrp4 to mediate ligand binding [42].

Dimerization of RTKs leads to activation of intracellular tyrosine kinase domain (TKD) through receptor-specific mechanisms. This variation in the activation mechanisms of TKD is largely due to structurally differences of inactive TKDs between different RTKs. Unique structure of inactive TKDs causes each TKD to be uniquely *cis*-auto inhibited and grants each RTKs to have their own regulatory mechanisms

(involving set of intramolecular interactions) [42]. On the other hand, the structure of activated forms of TKDs of RTKs are very conserved; they have key regulatory elements including the 'activation loop' and the kinase N-lobe adopts a specific configuration for catalysis of phosphor-transfer [46]. Following the RTK dimerization/oligomerization, auto-phosphorylation of receptors themselves occur subsequently. Auto-phosphorylation on the kinase domain of RTKS play an important regulatory role on receptor functions (except for EGFR and Ret) as it significantly impacts the enzymatic activity of the kinase domain. For example, auto-phosphorylation of the activation loop in insulin receptor TKD increases its catalytic capacity by 50-200 fold [47]. Geometrically, auto-phosphorylations occur in *trans* conformation, in contrast to RTK auto-inhibitions which usually occur in *cis* configuration [48, 49]. Furthermore, following the initial auto-phosphorylation events (primarily to increase its catalytic activity), other tyrosine residues on the cytoplasmic region of RTKs are also auto-phosphorylated. These secondary auto-phosphorylation events are crucial for recruitment and activation of downstream signaling proteins [50]. The resulting phospho-tyrosines on RTKs function as binding sites for cytoplasmic signaling molecules containing Src homology-2 (SH2) or phosphotyrosine-binding domains (PTB) [51]. Recruitment can be direct or through binding to docking proteins that are phosphorylated by RTKs, which in process is analogous to secondary auto-phosphorylation. Examples of these docking proteins include Gab1 and IRS-1 [52, 53]. Structurally, these docking proteins contain a membrane targeting site at the N-terminus and multiple phosphorylation sites that serve as binding sites for signaling protein in the cytoplasm [42]. Whether the signaling proteins are directly recruited or through the involvement of docking proteins, it is clear that multiple phosphor-tyrosine sites on RTKs attract various signaling proteins, thus making RTKs as a central node for complex signaling network. The resulting RTK-based signaling are produced in an oscillating manner and this is due to presence of numerous positive and negative feedback loops. Positive feedback loops increase the sensitivity of the signaling by amplifying the stimulus while negative feedback loops act to dampen the noise, preventing stochastic fluctuations in signaling and promoting tolerance. Combination of positive and negative feedback loops result in characteristic oscillations in RTK-based signaling [42].

Due to their potent, diverse downstream signaling activities, RTK activities are tightly regulated in normal physiological condition. However, dysregulation of RTK activity can lead to various cancers. Structural alterations or mutations in RTKS can cause abnormal activation and subsequently promote development and progression of various cancers. Specifically, dysregulation of RTK activities result in disruption of balance between cell proliferation and cell death [50]. Constitutive activation of RTKs occur through one of four principal mechanisms: 1) overexpression 2) gain-of-function mutations 3) chromosomal rearrangements 4) autocrine activation [50]. Overexpression of RTKs results in increased

concentration of receptors on cell surface, which results in elevated signaling that overpowers against antagonistic regulatory pathways. Although a majority of RTK overexpression occurs through genomic amplification, additional mechanisms include oncogenic viruses, loss of phosphatase, and transcriptional/translational enhancement [54-56]. Examples of overexpression include *EGFR* in glioblastoma, lung cancer and thyroid cancer [57-59], *HER2/ErbB2* in lung cancer [60] and *MET* in gastric cancer [61]. Gain of function mutations are another way that RTKs can constitutively be activated. These mutations are known to confer growth advantages to cells baring them and are known as 'driver mutations' [62]. Somatic *EGFR* mutations are excellent examples of gain of function mutations that confers oncogenic properties. Majority of these mutations are in-frame deletions or point mutations in exons of tyrosine kinase domain (although mutations can occur within extracellular domain and transmembrane domain of *EGFR*), and resulting mutations hyper-activate the kinase [63-66]. Furthermore, oncogenic properties of RTKs can be conferred through formation of novel tyrosine kinase fusion onco-proteins via chromosomal rearrangements. Chromosomal rearrangements join components of normal RTKs (can be fused as a whole intact protein or just tyrosine kinase domain based on the genomic breakpoint) with other proteins (fusion partners) through either N-terminal or C-terminal end of RTKs. Interestingly, fusion RTK onco-proteins can occur with multiple fusion partners even in the case of same disease [67, 68]. For example, *ALK* is known to have various fusion partners through gene rearrangement in non-small cell lung cancer (NSCLC) such as *EML4, TFG, KLC1, PTPN3, HIP1, TPR, DCTN1, PPM1B* [69]. Different fusion partners are known to play different biological roles that influence drug sensitivity and response [70]. Despite their varying effects on treatment sensitivity, all fusion partners share various features such as regulating the expression of fusion proteins, promoting oligomerization (which allows ligand independent activation of the kinase domain), and controlling subcellular localization of the protein [71-74]. Lastly, constitutive activation of RTKs can be achieved by continuously secreting ligands (such as growth factors and cytokines). Constitutive autocrine activation has been well documented in various cancers such as SCF-KIT autocrine loops in Merkel cell carcinoma (MCC) and small cell lung cancer (SCLC) [75, 76]. By producing large amounts of the ligand, the corresponding RTKs can remain activated and subsequently promote tumor development [77]. In addition to these four main mechanisms, other emerging mechanisms have been proposed in recent years including microRNAs, alterations in tumor microenvironment and signal attenuation by negative regulators [78-80]. Oncogenic properties of RTKs in cancer cells can be prevented by selective tyrosine kinase inhibitors (like small molecule inhibitors, monoclonal antibodies, heat shock proteins, immune-conjugates, peptide drugs) and thus act as promising therapeutic targets in various cancers [81]. Examples of these inhibitors include

cetuximab (monoclonal antibody that interferes with RTK activation) in lung cancer, imatinib (Gleevec) in chronic myelogenous leukemia, and trastuzumab (Herceptin) in advanced breast cancer [82, 83].

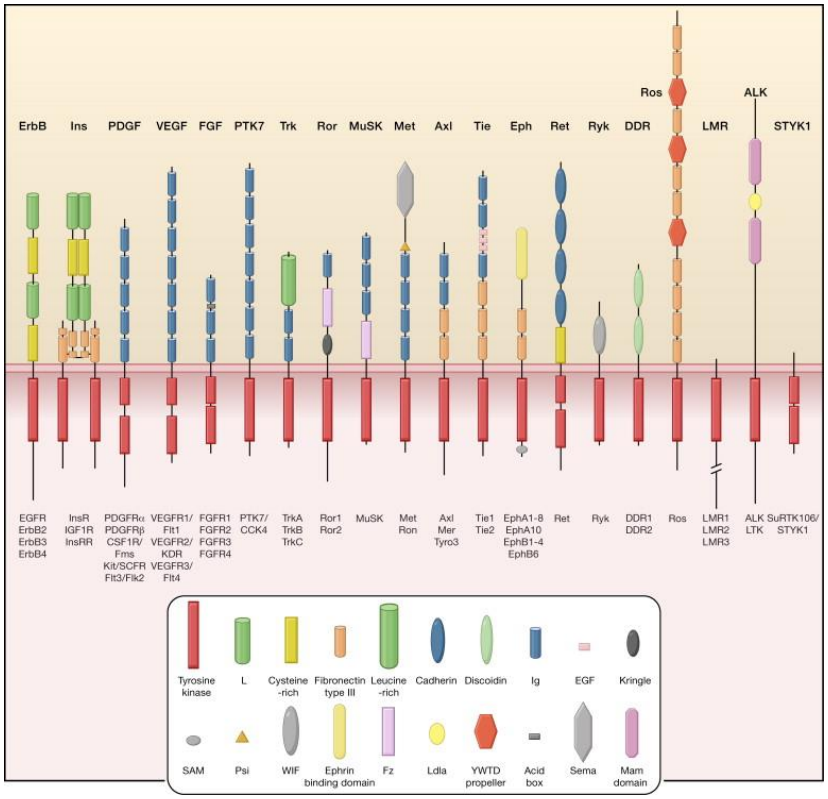


Figure 1. 5. Human RTKs. There are total of 58 human RTKs that belong to 20 subfamilies. This figure was extracted from Lemmon et al. (Cell Signaling by Receptor Tyrosine Kinases, Cell, 2010) with the permission granted by Rightslink (Elsevier).

1.2.1. Biology and function of ROR2

ROR2 is a member of RTKs that belongs to a family of orphan receptor tyrosine kinases (Ror) that comprises of two members in mammals, ROR1 and ROR2. ROR receptors are closely related to Trk neurotropin receptors and muscle-specific kinase [84]. Functionally, ROR2 plays vital roles in developmental morphogenesis, specifically in development of bones, cartilage growth plates, and digits [85] . During embryo development, ROR2 displays a distinct spatiotemporal expression patterns [86]. Its importance in developmental biology is highlighted in both mice and human lacking ROR2. For example, mice lacking ROR2 exhibit several developmental deficiencies such as dwarfism, ventricular septal defects, respiratory dysfunction, short limbs and tails, and facial abnormalities [87]. In humans, non-functional

mutations in *Ror2* result in brachydactyly type B, a condition characterized by undersized stature, shortening of the limbs, spinal defects and dysmorphic facial features [88, 89]. Structure of ROR2 is evolutionarily conserved like other RTKs although it is missing several key conserved amino acids typical of RTKs [85]. ROR2 consists of three major parts like other RTKs: extracellular, transmembrane and intracellular regions. The extracellular region is divided into immunoglobulin (Ig)-like domain, the cysteine rich domain (CRD), and the Kringle domain. Ig-like domain consists of ~100 amino acid residues, including a conserved disulfide bridge, and is thought to be important in mediating protein-protein interactions and functional modifications of CRD and Kringle [90]. ROR2 possesses a single CRD domain (defined by 10 conserved cysteines and several additional conserved amino acids) which is composed mainly of α-helices and acts as a ligand binding site [90]. Kringle domain is characterized by the presence of a triple-disulfide domain and functions as a recognition molecule for other proteins [90]. The cytoplasmic region consists of a putative tyrosine kinase domain along with proline rich domains as well as serine/threonine rich regions, the function of which are unknown [90]. The putative tyrosine kinase domain contains a conserved tyrosine containing motif (YALM) that has potential to bind to SH2 domains or p85 subunit (ie. Phosphoinositide 3-Kinase) of various downstream mediators upon phosphorylation [90]. The proline rich domain in the cytoplasmic tail of ROR2 serves as a docking structure [90]. **Figure 1.6** depicts a general structure of ROR2.

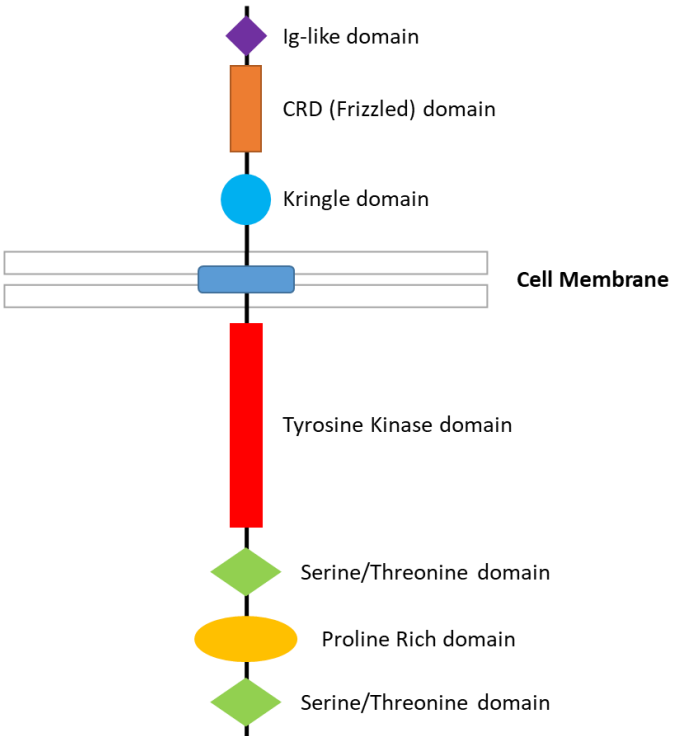


Figure 1. 6. Structure of ROR2. Extracellular region of ROR2 contains Ig-like domain, a CRD (Frizzled) domain, and a Kringle Domain. CRD domain acts as a binding site for Wnt ligands. Extracellular region is separated from the intracellular region by a transmembrane (TM) domain that runs through the cell membrane. The intracellular region contains a tyrosine kinase domain and a proline-rich domain bordered by two Serine/Threonine rich domains.

Molecularly, ROR2 is a mediator of non-canonical Wnt signaling pathway. Wnts are highly conserved, secreted glycoproteins that mediate various biological processes vital for development and homeostasis (such as morphogenesis, patterning, and lineage decision) by activating either canonical (Wnt/β-Catenin dependent) pathway or non-canonical (Wnt/β-Catenin independent) pathway. [91]. Major difference between canonical and non-canonical pathways is the involvement of β-Catenin. Activation of canonical Wnt pathway results in stabilization and accumulation of β-Catenin through disruption of a protein complex (consists of GSK3β, APC, Axin) that normally degrades β-Catenin. β-Catenin is then transported into the nucleus and act as a coactivator of various transcription factors belonging to TCF/LEF family [92]. On the other hand, non-canonical pathway consists of downstream effectors other than β-Catenin-TCF/LEF [93]. Examples of these downstream effectors include c-Jun N-terminal kinase (JNK) and phospholipase C (PLC) [93]. Canonical pathway predominantly regulates

transcriptional responses in the cell while non-canonical pathways regulate both transcriptional and non-transcriptional responses. Which of the two pathways is activated depends on the Wnt/receptor combination as well as the cell types as there are numerous Wnt ligands and corresponding receptors (ie. Frizzled and low-density lipoprotein receptor-related protein (LRP)) [94]. The non-canonical Wnt pathway is involved in various different cellular functions including planar cell polarity, convergent extension, calcium fluxes, and cytoskeletal rearrangements and ROR2 mediates its signaling by acting as a receptor for Wnt5a [95]. Although Wnt3 is also a known ligand for ROR2, only Wnt5a is shown to induce ROR2 activity [92]. The involvement of ROR2 in Wnt5a-mediated signaling as its receptor has been supported by display of strong phenotypic similarities between mice deficient in ROR2 and Wnt5a, as well as having overlapping spatiotemporal expression patterns between ROR2 and Wnt5a in developing embryo [86, 96, 97]. Furthermore, multiple models have shown that ROR2-mediated Wnt5a signaling results in antagonistic inhibition of canonical Wnt pathway, although it is suggested that ROR2 can also promote canonical signaling [98-101]. Various signaling partners and cellular functions of Wnt5a/Ror2 axis have been established based on phenotypic defects in *Caenorhabditis elegans* and other vertebrate models [94]. For instance, Wnt5a-ROR2 signalling increases receptor activator of nuclear factor-κB (RANK) expression in osteoclast precursors by activating JNK and recruiting c-Jun to RANK promoter, promoting osteoclastogenesis [102]. Several other intracellular signaling partners have been identified besides JNK, such as ERK and c-Src, and other downstream signaling mechanisms have been discussed [85, 103-105]. ROR2 mediates Wnt5a signaling through a classical RTK-like fashion (ie. ligand binding, dimerization, activation of tyrosine kinase) although much of the evidence for kinase activity has been indirect [85, 90]. It is believed that despite of its minimal catalytic activity, the kinase activity that ROR2 possesses is still sufficient to carry out its function. Furthermore, ROR2 may function as a scaffold protein by allosterically activating a functional kinase [90]. ROR2 is known to form homo-dimers as well as hetero-dimers with other partners such as ROR1 [106]. In addition to binding to its main ligand Wnt5a, there is growing evidence that ROR2 binds to other Wnt ligands such as Wnt7, Wnt8, and Wnt11 [107].

1.2.3. Role of ROR2 in cancer

ROR2 has been documented to play a pivotal role in various different cancers such as colon cancer, hepatocellular carcinoma, metastatic melanoma and renal cell carcinoma [108]. Due to its pleiotropic nature, ROR2 possesses dual roles and acts to either suppress or promote carcinogenesis [108]. The upregulation and oncogenic role of ROR2 has been established in a multitude of tumour types including prostate cancer, osteosarcoma, melanoma, renal cell carcinoma and stromal tumours [108]. While in

certain cancer types such as osteosarcoma, the upregulation of ROR2 is throughout the majority of cancer cells, ROR2 overexpression is specific to metastasis and cancer dormancy in cancers such as prostate cancer and melanoma [109-111]. Molecularly, the oncogenic role of ROR2 is focused on promoting cell migration and tumorigenic invasion. For example, ROR2 signaling activates and maintains cell dormancy of prostate cancer in bone metastasis by inducing SIAH2 expression while promoting cell migration in renal cell carcinoma by inducing expression of epithelial-mesenchymal transition (EMT) associated genes such as Twist and MMP2 [111, 112]. In contrast to its tumorigenic properties, the role of ROR2 as a tumor suppressor has been elucidated in various studies in colon cancer, hepatocellular cancer and hematological malignancies [108]. For example, reduced expression of ROR2 is observed in colorectal cancer and hepatocellular carcinoma [108]. Molecularly, the role of ROR2 as a tumor suppressor stems from its ability to inhibit canonical Wnt signaling. For instance, canonical Wnt signaling pathway is hyper-activated in colorectal cancer and inhibition of canonical Wnt pathway via restoration of ROR2 expression reduced tumor formation [113]. The dual role of ROR2 in tumorigenesis mainly depends on the type of Wnt signaling pathways activated in tumour development; in cancers driven by canonical Wnt signaling, ROR2 activity will hinder tumour growth whereas in cancers driven by non-canonical Wnt signaling, more critical and direct role of ROR2 is observed in driving tumorigenesis [92].

Due to its limited expression pattern in normal adult and its role in tumorigenesis in certain cancer types, ROR2 represents an extremely attractive target. There are several small molecule tyrosine kinase inhibitors for various cancers that target the ATP-binding site of intracellular TKD [83]. Unfortunately, due to the possibility of ROR2's TKD being minimally functional, ROR2 is not subjected as a target for these small molecule inhibitors. Instead, alternative therapeutic strategies such as monoclonal antibodies that bind and neutralize ROR2 or conjugate antibodies that deliver potent toxins can be utilized to target ROR2 [92].

1.3. Programmed Death-Ligand 1 (PD-L1/CD274)

1.3.1. Biology and function

The adaptive immune system has evolved to recognize and destroy aberrant cells such as pathogen-infected cells and cancer cells and amongst various immune cells involved, T cells play a major part in coordinating various aspects of the immune system. T cells recognize their target cells through binding of their T cell receptor (TCR) to major histocompatibility complexes (MHC)-peptide complex on the recipients cells [114]. Based on the peptide present in the MHC complex, T cells can differentiate between the normal cells and aberrant cells and selectively destruct the abnormal cells. To further promote self-tolerance and reduce the risk of potential autoimmunity, T cell interactions are tightly regulated through various arrays of co-stimulatory and co-inhibitory receptors and complementary ligands (known as the immune checkpoints). Based on these immune checkpoint interactions, T cell activity can be activated or suppressed even with the presence of TCR-MHC complex binding [114]. Among different immune checkpoints, PD-L1-PD-1 axis is one of the negative regulators of T cell activity that has stood out, partly due to its role in conferring immune evasion in various cancers. PD-L1-PD-1 axis acts as a critical determinant of immune homeostasis; PD-1 deficient mice of different genetic backgrounds are prone to develop lupus-like autoimmune disease or fatal cardiomyopathy while PD-L1 blockade has shown to impair fetomaternal tolerance [114]. PD-L1 is one of the two known ligands (other one being PD-L2) that interacts with co-inhibitory receptor PD-1 expressed on the surface of antigen-stimulated T cells [115]. While PD-L2 is conventionally present on small subset of immune cells (macrophages, mast cells, and dendritic cells), PD-L1 expression profile is much more diverse; PD-L1 expression can be detected in hematopoietic cells such as T cells, B cells, macrophages, dendritic cells, and mast cells, as well as in non-hematopoietic cells in the tissues such as vascular endothelial cells, keratinocytes, pancreatic islet cells, astrocytes, and others [114]. PD-L1 is a type I transmembrane protein that belongs to immunoglobulin (Ig) superfamily. Structurally, it contains an extracellular domain composed of Ig variable (Ig-V) distal region and Ig constant (Ig-C) proximal region, a transmembrane domain, and an intracellular cytoplasmic tail with three conserved motifs (RMLDVEKC, DTSSK, and QFEET) [114, 116]. The Ig-V region of the extracellular domain presents a standard Ig-like domain with complementary determining-like regions (CDRs) that acts as a binding domain for PD-1 in a 1:1 stoichiometric ratio, similar to the antigen recognition mechanism by antibodies and T cell receptors (TCRs) [114, 116]. Interactions between the extracellular domains of PD-L1 and PD-1 induce a conformational change in PD-1 and subsequent activation of downstream pathways mediated by Src family kinases; PD-L1 bound PD-1 is subjected to phosphorylation at two immunological cytoplasmic motifs, immunoreceptor tyrosine-based inhibitory

motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM). Following their phosphorylation, these motifs recruit phosphatases (SHP-1 and SHP-2) that attenuate T cell activating signals and reducing T cell activity by inhibiting T cell proliferation, survival, cytokine production, and other effector functions [114]. In addition to its ability to interact with main receptor PD-1, PD-L1 can also interact with co-stimulatory molecule CD80 and deliver inhibitory signals to activated T cells through an alternative route [114]. Apart from the membrane-bound PD-L1, PD-L1 can be found in various other forms. These include cytoplasmic PD-L1, nuclear PD-L1, and serum PD-L1 [117]. The structures of these PD-L1 are versatile as well, with some lacking transmembrane motifs and the potential of glycosylation and dimerization [117]. **Figure 1.7** depicts a general molecular structure of PD-L1.

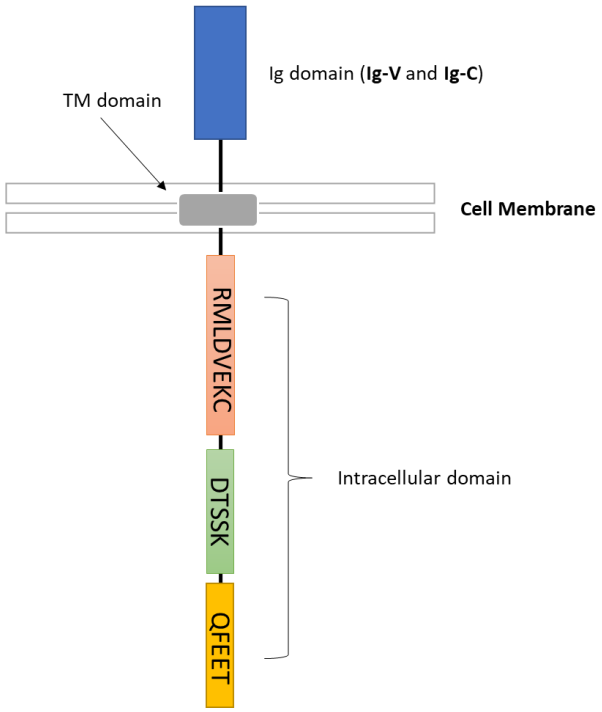


Figure 1. 7. Structure of PD-L1. Extracellular region of PD-L1 is an Ig domain that contains Ig-V and Ig-C regions. Extracellular region is separated from the intracellular region by a transmembrane (TM) domain that runs through the cell membrane. The intracellular region of PD-L1 consists of three conserved motifs, RMLDVEKC, DTSSK, and QFEET.

1.3.2. Role of PD-L1 in cancer

Various cancers use PD-L1 overexpression as an evading mechanism from body's anti-tumor immune system lead by T cells. It has become clear that virtually all cancer cells express tumour-specific and tumour selective antigens that are generated as a result of genetic alterations and epigenetic dysregulation. These tumour antigens make cancer cells very immunogenic and the immune system eliminates these cancer cells, resulting in strong selection for poorly immunogenic or immune-resistant cancer cells (this process is known as immune-editing). There are various mechanisms of how the cancer cells become immune evasive. One of the major ways that the tumor cells can acquire immunosuppression is through overexpression of PD-L1. Upregulation of PD-L1 expression results in chronic PD-L1 engagement with PD-1 on T cells. This constant binding of PD-L1 to PD-1, in turn, causes progressive loss of T cell effector functions, eventually reaching an exhausted (or dysfunctional) state [114]. Exhausted T cells, characterized by high expression of PD-1, lose their capacity to eliminate tumour cells and are frequently observed within tumours and in peripheral blood [118]. Various molecular mechanisms of PD-L1 upregulation have been reported in different cancers; these include **genomic alterations** (ie. gene amplification, translocation in chromosome 9p24.1, and structural alteration in 3'-untranslated region), **epigenetic changes** (ie. bromodomains and extraterminal (BET) proteins, histone acetylation and methylation), **transcriptional regulation** (ie. IFNγ-JAK-STAT pathway, NF-κB pathway, HIF-1, c-Myc, PI3K pathway, EGFR pathway, Hippo signaling), **microRNAs** (ie. miR-200, miR-34a), and **post-translational modifications** (ie. mono- and poly-ubiquitination of PD-L1, PD-L1 phosphorylation, production of PD-L1 binding partners such as CMTM6, and glycosylation). Apart from intratumoural upregulation of PD-L1, recent study from Kim et al. suggests that inducing exosomal expression of PD-L1 also contributed to immunosuppression and growth by cancer cells [119]. Furthermore, secreted serum PD-L1 (and PD-L1 variants) have been reported to contribute to immune evasion in certain cancers (ie. NSCLC) [120]. Based on their influence as a major mechanism of immunosuppression in cancer, numerous studies have been made to validate the prognostic significance of PD-L1 in different cancer types. For example, PD-L1 overexpression is a prognostic biomarker in renal carcinoma and urothelial cancer with higher expression correlating with poor survival [121].

Given their immense role in immune evasion for various cancer types, PD-L1 and PD-1 have been identified as prominent targets for cancer immunotherapy and antibody-based PD-L1/PD-1 inhibitors were developed [122]. Although various studies have reported therapeutic potentials of PD-1/PD-L1 blockade in pre-clinical models, the first clinical data supporting the efficacy of PD-1 was reported by Brahmer and his colleagues in 2010; monoclonal antibody-based PD-1 blocking agents showed antitumor

activity with acceptable toxicity profile in patients with colorectal cancer, melanoma, and renal cell carcinoma [123]. Subsequent studies have further confirmed significant antitumor activity of PD-L1/PD-1 blockade in various cancers, leading to the development of first clinically available PD-1 inhibitors, pembrolizumab and nivolumab, approved by FDA in 2014. Since the approval of pembrolizumab and nivolumab for treating advanced melanoma, the scope of clinical development of PD-1/PD-L1 inhibitors have broadened. Currently, there are total of six anti-PD-L1/PD-1 agents (pembrolizumab, nivolumab, avelumab, atezolizumab, cemiplimab and durvalumab) clinically approved for thirteen different types of cancers (melanoma, NSCLC, triple-negative breast cancer, urothelial carcinoma, SCLC, HNSCC, Hodgkin's lymphoma, MSI-H and dMMR colorectal cancers, Merkel-cell carcinoma, gastric cancer, cervical cancer, CSCC, and renal cell carcinoma) [114, 124]. **Table 1** summarizes the cancer types and applicable FDA approved PD-1/PD-L1 antibody products [117].

Table 1. **Cancer types and corresponding FDA approved PD-L1/PD-1 based therapy**

Cancer Types	**FDA Approved PD-L1/PD-1 based therapy**
Melanoma	Nivolumab, Pembrolizumab
Urothelial Carcinoma	Atezolizumab, Durvalumab, Nivolumab, Pembrolizumab
NSCLC	Atezolizumab, Nivolumab, Pembrolizumab
SCLC	Nivolumab, Atezolizumab
TNBC	Atezolizumab
Merkel cell Carcinoma	Avelumab
Hodgkin's lymphoma	Nivolumab, Pembrolizumab
Renal cell carcinoma	Nivolumab
HNSCC	Nivolumab, Pembrolizumab
Cervical Cancer	Pembrolizumab
Gastric Cancer	Pembrolizumab
CSCC	Cemiplimab
MSI-H and dMMR colorectal cancers	Nivolumab, Pembrolizumab

Apart from antibody-based blockade, other therapeutic approaches to target PD-L1/Pd-1 axis have been made. The rationale to investigate other forms of therapy stems from the observation that antibody-based therapy is primarily efficient against membrane-bound PD-L1 and has limited influence on intracellular forms. These include gene silencing and small-molecule pathway inhibition [117]. The gene silencing approach utilizes small interfering RNA or microRNAs to induce knockdown of PD-L1 directly in tumour cells [117]. Several in vitro studies have demonstrated the efficacy of RNA-based knockdown in sensitizing tumour cells to T cell-based killing [125, 126]. However, due to relatively short lifespan and vulnerability to circulating RNases, delivery is the main limitation of RNA-based gene silencing

[117]. The small-molecule pathway inhibition uses small molecules to target relevant expression pathways (ie. regulatory signaling pathways). PD-L1 expression is mainly regulated via MAPK and PI3K/Akt pathways and there have been efforts made to develop specific inhibitors for these pathways. Unfortunately, there is no direct evidence that support this approach to date. However, the idea of using small molecules remains as an attractive option due to its effectiveness in internalizing into target cells.

1.4. Hypothesis Formation

Various RTKs have been reported to have oncogenic roles in various cancers, including prostate cancer. One of its members, ROR2, has been identified to play a role in prostate cancer, particularly in promoting metastasis and inducing cancer cell dormancy. While the oncogenic role of ROR2 has been elucidated in various human prostate cancer models (ie. LNCaP, DU145, PC3, and 22RV1), whether ROR2 plays a role in the pathogenesis of treatment-resistant prostate cancer is still unknown. Even with the recent development of potent antiandrogens such as Enzalutamide and Abiraterone, treatment resistance is inevitable in a majority of cases and thus, understanding the molecular mechanism of treatment-resistant prostate cancer is essential in identifying potential therapeutic targets.

1.4.1. Preliminary data

Our laboratory has generated CRPC and ENZ-resistant (ENZ^R) CRPC cell lines through serial passaging of LNCaP-derived xenografts in mice [127, 128]. In order to gain insights on the molecular changes that occur during the progression of CRPC to treatment-resistant form, we performed RNA-sequencing (RNA-seq) in parental CRPC cell line (16D) and ENZ^R CRPC cell line (42D) and analyzed the gene expression patterns. With a particular interest in RTKs, we compared the expression of all known human RTKs between 16D and 42D and ranked them based on the fold change. We found that ROR2 is one of the top RTKs that is upregulated in 42D compared to 16D (**Figure 1.8A).** This observation was consistent when we compared RNA sequencing data between pre-castration (pre-CX) and post-castration (post-CX; 8 weeks) in Patient Derived Xenograft (PDX) mice models (**Figure 1.8B)**. To further investigate whether our in vitro observation was consistently translated into the clinical model, we accessed publicly available patient dataset (cBioPortal) and compared the expression levels of top four upregulated RTKs. Interestingly, ROR2 was the only highly RTK expressed in CRPC patients amongst the examined RTK and its expression was even further elevated in NEPC patients, indicating a potential of clinical significance (**Figure 1.8C**)

C

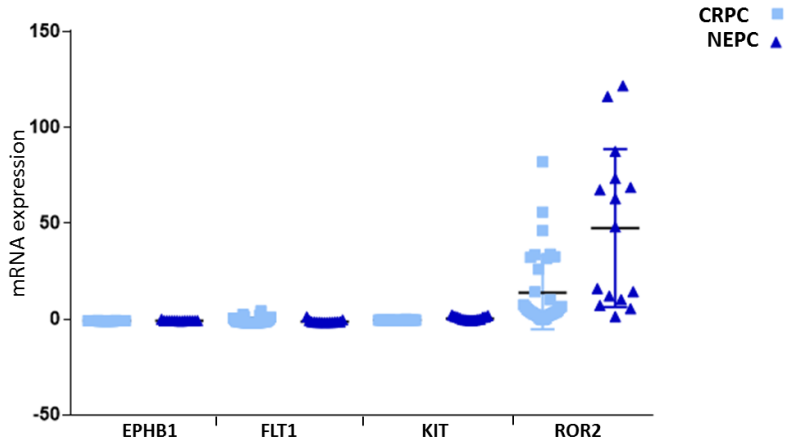


Figure 1. 8. ROR2 is upregulated in treatment-resistant prostate cancer. (A, B) all human RTKs are ranked based on the fold change in mRNA expression of RNA sequencing datasets **(A)** 42D vs 16D **(B)** post-castration (post-CX) vs pre-CX in PDX. **(C)** mRNA expression of upregulated RTKs observed in in vitro treatment-resistant cell line (42D vs 16D) in CRPC and NEPC patients (Multi-institute, 2016).

1.4.2. Hypothesis

RNA-sequencing based gene expression profiling revealed that ROR2 is one of the top RTKs that is highly upregulated in treatment-resistant models (42D and post-CX PDX) compared to their parental models. Therefore, **we hypothesize that ROR2 plays a role in treatment-resistant prostate cancer.**

2. Materials and Methods

2.1 Cell Culture

Enzalutamide (ENZ) sensitive CRPC cell line 16D was generated from *in vivo* serial passaging of prostate adenocarcinoma (PCa) and ENZ-resistant CRPC cell lines were derived from *in vivo* passaging of CRPC tumor under ENZ. Castration-resistant prostate cancer (CRPC) cell line V16D were maintained in RPMI-1640 (ThermoFisher) media supplemented with 10% FBS (Invitrogen), and Enzalutamide (ENZ)-resistant CRPC cell lines 42D and 42F were grown in 10% FBS RPMI-1640 media with 10µM Enzalutamide (Selleck Chemicals).

2.2 Transfections

For small interfering RNA transfections (transient loss of function experiments), V16D, 42D and 42F were transfected with control siRNA (siScr) or ROR2 siRNA (siROR2) at final concentration of 20nM using Lipofectamine 3000 (ThermoFisher) according to the manufacturer's protocol.

Stable ROR2 knockdown 42D and 42F cell lines were generated by using ROR2 small hairpin RNA (shROR2). Lentivirus particles containing control vector and shROR2 were generated using manufacturer's protocol and were used to transfect target cell lines. After 72 hours of transfection, the cells were selected by growing them in 10% FBS RPMI-1640 media with 1% puromycin (Gibco) and 10µM Enzalutamide (Selleck Chemicals). Stable knock-down of ROR2 was achieved using U6-shROR2-Puro purchased from Vector Builder.

For transient ROR2 over-expression in V16D, empty vector and wild-type human ROR2 vector were used. Cells were seeded at 1 x 10^6 cells in 10 cm^2 dishes and transfected using *Trans*IT-2020 (Mirus) based on the manufacturer's protocol. Over-expression of ROR2 was achieved using pEF1a-mRor2WT purchased from Addgene (ID: 22613).

2.3 Western Blotting

Total protein was extracted from each cell line using RIPA lysis buffer containing phosphatase inhibitor (PhosSTOP, Millipore-Sigma) and protease inhibitor cocktail (Roche). Protein concentration was determined using Pierce BCA Protein Assay Kit (ThermoFisher). Samples were prepared by mixing protein extracts with water and 4x sample buffer. Protein was separated on 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane overnight at 4°C. The membrane was probed with primary antibodies

overnight at 4°C at 1:1000 dilution prepared in 2.5% Bovine Serum Albumin (BSA) TBS-T solution. The membrane was visualized using Odyssey Infrared Imaging System (Li-COR Biosciences).

2.4 Flow Cytometry

Cells were removed from 10cm^2 dishes by using 5mL of Corning™ CellStripper Dissociation Reagent (ThermoFisher) for 10 minutes at room temperature. Once detached, cells were incubated with fluorchrome-conjugated antibodies at 1:50 dilution in FACS buffer (2% FBS, 1mM EDTA, 0.1% Sodium Azide in PBS) for 30 minutes at 4°C. Cells were washed with FACS buffer and suspended in 200µL of FACS buffer. Analysis was done using BD FACSCanto II cytometer (BD Biosciences).

2.5 Quantitative Real Time PCR (qRT-PCR)

RNA was extracted from cells in 10cm^2 dishes using TRIzol reagent (ThermoFisher). Extracted RNA pellet was washed with 70% ethanol and re-suspended in nuclease-free water (Invitrogen). RNA concentration was measured by using NanoDrop™ 2000 Spectrophotometers (ThermoFisher). cDNA was synthesized using 2µg of RNA and M-MLV Reverse Transcriptase Kit (ThermoFisher). Quantitative real time PCR amplification of synthesized cDNA was performed using FastStart Universal SYBR Green Master (Roche) with custom primers for GAPDH, ROR2, Wnt5a, CD274, CD44, AR, PSA, and CD133. Gene expression was normalized to GAPDH and all experiments were performed in triplicates.

2.6 Gene Set Enrichment Analysis (GSEA)

GSEA is a computational method to determine whether sets of genes (grouped into relevant pathways) show statistically significant, concordant differences (either enriched or downregulated) between two biological states (ie. phenotypes) [129]. GSEA allows the user to compare different datasets such as RNA sequencing data. GSEA was performed by first converting the data files to appropriate formats (ie. gct, cls, txt). Then, we loaded the data files into GSEA software and set the analysis parameters (these parameters were sample-specific and were customized to each sample). Once the parameters were set, we ran the analysis.

2.7 Gene Expression Profiling

<u>2.7.1 RNA-sequencing (RNA-seq):</u> Samples for RNA-seq were prepared using TRIzol reagent (ThermoFisher) and the RNA quality was measured using NanoDrop (ThermoFisher). Transcriptome sequencing was performed using Illumina HiSeq 2000 (illumina) according to manufacturer's protocol.

Sequencing data mapping and processing was performed as previously described [130]. Quantification of gene expression was performed by RSEQtools using GENCODE v19 as reference gene annotation set. Gene expression was represented as RPKM (Reads Per Kilobase of transcript per Million mapped reads).

2.9 Analysis of Public Databases

CBioportal for Cancer Genomics: This website allows the researcher to visualize, download and analyze large-scale cancer studies such as The Cancer Genome Atlas (TCGA) studies for different cancers [131]. Expression dataset (mRNA expression in z-score format) from TCGA provisional study for prostate adenocarcinoma (2015) and SU2C/PCF Dream Team study for metastatic prostate cancer (2015) were downloaded and used for various analysis.

2.10 Reagents and Antibodies

Antibodies against ROR2 (#4105S) and CD274 (#15165) were purchased from Cell Signaling Technology, USA. PECy7-conjugated antibody from CD274 (#374506) was purchased from Biolegend.

2.11 Statistical Analyses

P-values were calculated using Student t-test to compare control and treated groups and p-values less than 0.05 were considered statistically significant (*$P < 0.05$, **$P < 0.001$, ***$P < 0.001$).

3. Results

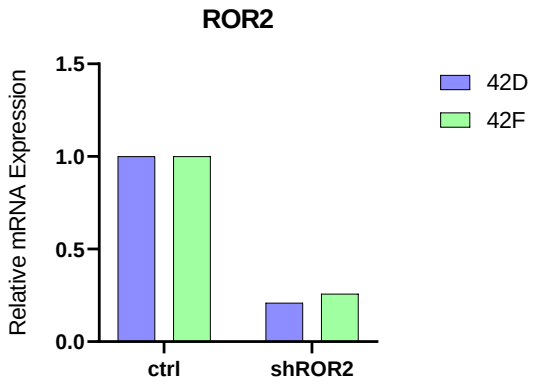
3.1. Generation of Custom ROR2 Gene Signature

The oncogenic role of ROR2 has been, in part, described in prostate cancer; however, the role of ROR2 is still unknown in treatment-resistant prostate cancer. From our preliminary data, we became aware that ROR2 expression is elevated during treatment resistance and this observation was consistent in patients as well. In order to investigate ROR2's function in treatment-resistant prostate cancer, we generated a novel ROR2 gene signature. The advantage of creating the novel ROR2 gene signature was to eliminate any bias and summarize the function of ROR2 in a molecular pattern.

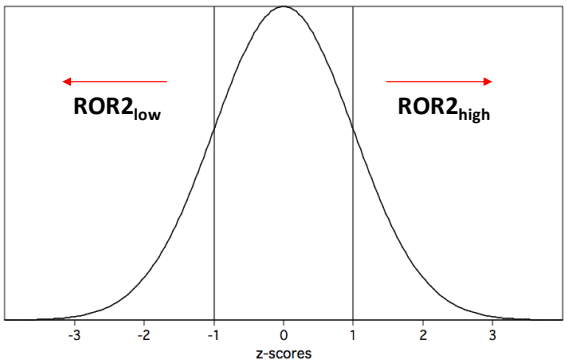
To generate cell lines of identical background but with differential ROR2 expression, we constructed stable ROR2 knockdown cell lines (shROR2 cell lines) in ENZ-resistant cell lines 42D and 42F, using shROR2. We validated successful knockdown by examining the mRNA expression level of ROR2 in stable knockdown cell lines compared to their parental controls (**Figure 3.1A)**. Once the cell lines were generated, we performed RNA sequencing on the cell lines along with the controls. As a starting point for generating our score, we analyzed expression profile data sets (RNA-seq) of shROR2 knockdown cell lines (42D shROR2 and 42F shROR2) using GSEA software; we generated a list of genes that were downregulated in shROR2 cell lines compared to their controls (or in other words, enriched in controls compared to knockdown cell lines). GSEA analysis was carried out independently for each cell line (ie. 42D vs 42D shROR2, 42F vs 42F shROR2). Along with the expression profile data sets from our generated knockdown cell lines, we also analyzed publicly available patient data set that accurately resembled our model. The patient dataset we used was from Aggarwal and his colleagues, where they investigated patients with progressive, metastatic CRPC despite being on abiraterone and/or enzalutamide. In order to carry out GSEA analysis on Aggarwal dataset, we first separated the patient data into two populations based on ROR2 expression (z-score); $ROR2_{high}$ (patients with ROR2 expression higher than 1 standard deviation above the mean) and $ROR2_{low}$ (patients with ROR2 expression lower than 1 standard deviation below the mean) (**Figure 3.1B)**. GSEA analysis on two subsets of patient datasets, $ROR2_{high}$ and $ROR2_{low}$, revealed a list of genes that were enriched in patients with high ROR2 expression. Then, we overlapped the lists of ROR2-dependent enriched genes from three independent GSEA analysis (42D vs 42D shROR2, 42F vs 42F shROR2, $ROR2_{high}$ and $ROR2_{low}$ populations in Aggarwal 2018) and identified common genes present in all three analyses. This list of common genes, a putative ROR2 gene signature, included all the genes that were enriched in ROR2-high populations. However, the extent of upregulation for each gene was not measured and the putative gene signature did not take the level of enrichment into the selection

criteria. In order to resolve this issue, we first took the putative ROR2 gene signature into each data sets and ranked the genes based on the enrichment score (ES). ES reflects the degree to which a sample gene is overrepresented at the extremes (top or bottom) of the entire list; ES scale runs from -5 to +5 with negative values indicating down-regulation, positive values indicating enrichment, and score of 0 indicating no change in gene expression [129]. From ranked ES scoresheets, we chose the top 20% of the genes from each data set and superimposed them together to identify common genes. Resulting list of 91 genes generated a novel ROR2 gene signature that are consistently present in different models (in vitro and patient dataset) and are highly enriched with high ROR2 expression profiles (**Table 2).**

A



B



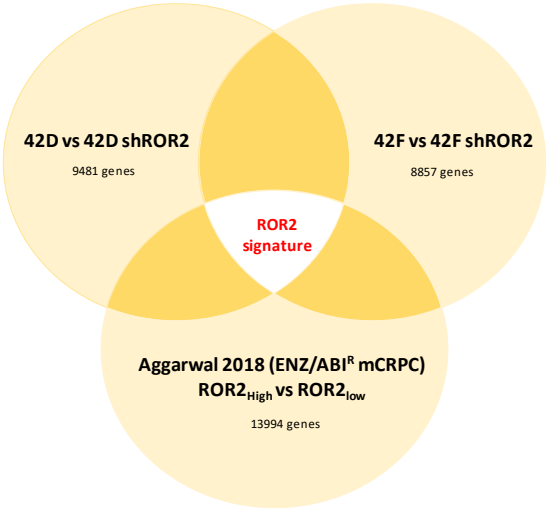


Figure 3. 1. Generation of ROR2 gene signature. A) relative mRNA expression of ROR2 in shROR2 knockdown cell lines (42D and42F) compared to their respective controls. **B)** Schematic representation of segregating Aggarwal dataset into $ROR2_{high}$ and $ROR2_{low}$ populations based on z-scores (**left**) overlapping of GSEA of three independent RNAseq datasets (**right**).

Table 2 List of genes in ROR2 gene signature

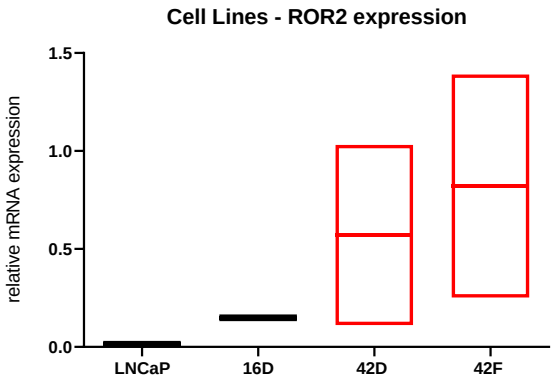
ACSL4	CDH18	IGSF11	PLSCR1	TBX22
ADM	CDH2	INSIG1	PMFBP1	TBX5
ANKFN1	CEACAM1	KCNE4	POPDC2	TENM4
ANKRD33B	CELF2	KCNV1	PRRT1	TLR6
ARMCX2	CFAP221	KIF26B	PTPRD	TMEM37
ATP10D	CYTIP	LST1	RAC2	TMEM45A
B3GNT8	DMRT3	MALRD1	RARB	TRANK1
BCAT1	DNAH3	MAML2	RCSD1	TRIM34
BEND6	DPEP1	MAST4	RUNX1	VSIG8
BHLHE22	DPY19L1	MDGA2	SAMD9	VSNL1
BRINP1	EFNA5	MPPED2	SCN3B	WDR72
C10orf131	FAM3C	MX2	SERPINH1	
C3orf58	FITM1	NAT1	SH3RF2	
C8orf48	GNG2	NDP	SLC39A8	
CALCB	GPX8	NNMT	SLC9A9	
CAPSL	GRIK2	NPR2	SLFN13	
CAV2	HOXA2	NTN3	SNAI2	
CCDC170	IER3	OLFML3	SOX7	
CD274	IFIT2	P3H2	ST8SIA4	
CD80	IFIT3	PLEKHA4	STARD4	

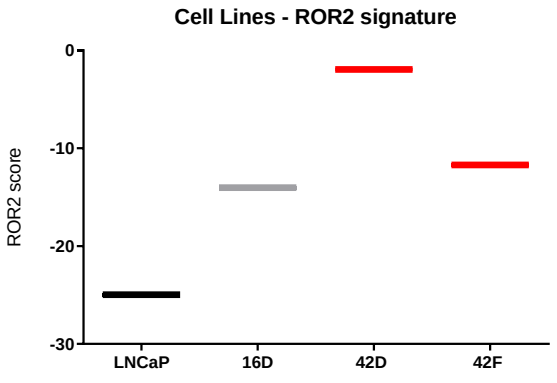
3.2. Validation of Custom ROR2 Gene Signature

Once the ROR2 gene signature was developed, we examined the ROR2 gene signature in various data sets to validate the gene signature. First, we examined RNA-seq data from available prostate cancer cell lines. We showed that ROR2 gene signature score was correlated with ROR2 expression in the cell lines and that ENZ-resistant cell lines (42D and 42F) had higher ROR2 expression and signature score compared to treatment-sensitive cell lines (LNCaP and 16D) (**Figure 3.2A**). Furthermore, this finding was consistent in two independent mouse models. The first mouse model dataset we utilized was Patient Derived Xenograft (PDX) prostate cancer model. This model was developed by implanting patient-derived cancer tissue specimens into immunodeficient mice (ie. Nude, SCID mice) and retains much of the heterogeneity, architectural and molecular characteristics of the original tumour and respective microenvironment [132]. Tissue sample was collected and sequenced at different stages of the disease. Our analysis of PDX model revealed elevated ROR2 expression and ROR2 signature score upon castration (**Figure 3.2B)**. In genetically engineered mouse models (GEMM) published by Ku and his colleagues, our analysis demonstrated that tumorigenic single knock-out model (SKO; *Pten* loss) and double knock-out model (DKO; *Pten* and *Rb1* loss) exhibited elevated ROR2 expression and score compared to non-tumorigenic wild type (WT) model (**Figure 3.2C**).

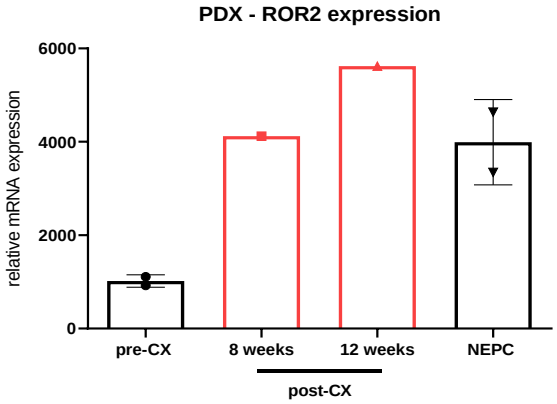
To validate the clinical relevance of the generated ROR2 gene signature, we investigated datasets from two independent patient cohorts (TCGA 2015 and SU2C 2015) along with Aggarwal dataset. As illustrated in **Figure 3.2D,** positive correlation between ROR2 expression and signature score in patients are consistent with and support the previous findings we observed in various in vitro and in vivo models. Our findings from various models (in vitro cell lines and mice) and patient cohorts validates and support the gene signature and its responsiveness to ROR2 activity.

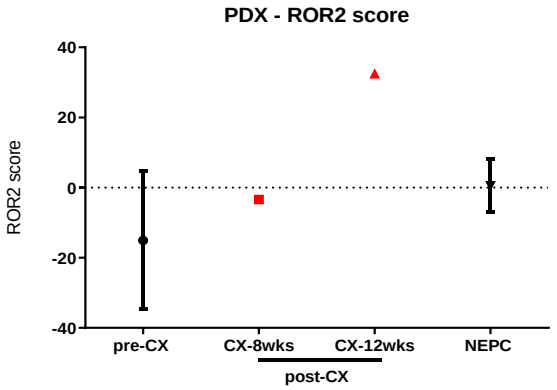
A


Cell Lines - ROR2 expression
relative mRNA expression
1.5
1.0
0.5
0.0
LNCaP
16D
42D
42F

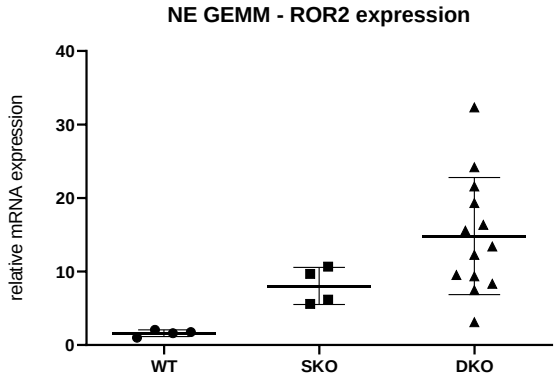

Cell Lines - ROR2 signature
ROR2 score
0
-10
-20
-30
LNCaP
16D
42D
42F

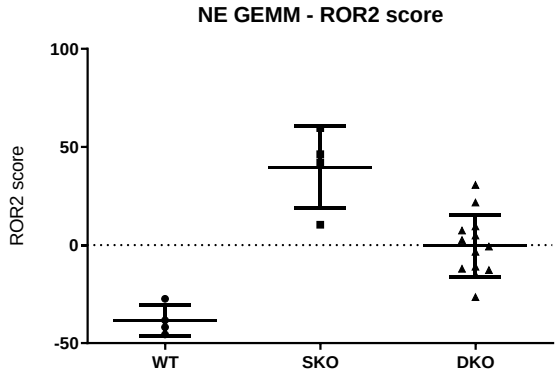
B


PDX - ROR2 expression
relative mRNA expression
6000
4000
2000
0
pre-CX
8 weeks
12 weeks
NEPC
post-CX


PDX - ROR2 score
ROR2 score
40
20
0
-20
-40
pre-CX
CX-8wks
CX-12wks
NEPC
post-CX

C


NE GEMM - ROR2 expression
relative mRNA expression
40
30
20
10
0
WT
SKO
DKO


NE GEMM - ROR2 score
ROR2 score
100
50
0
-50
WT
SKO
DKO

D

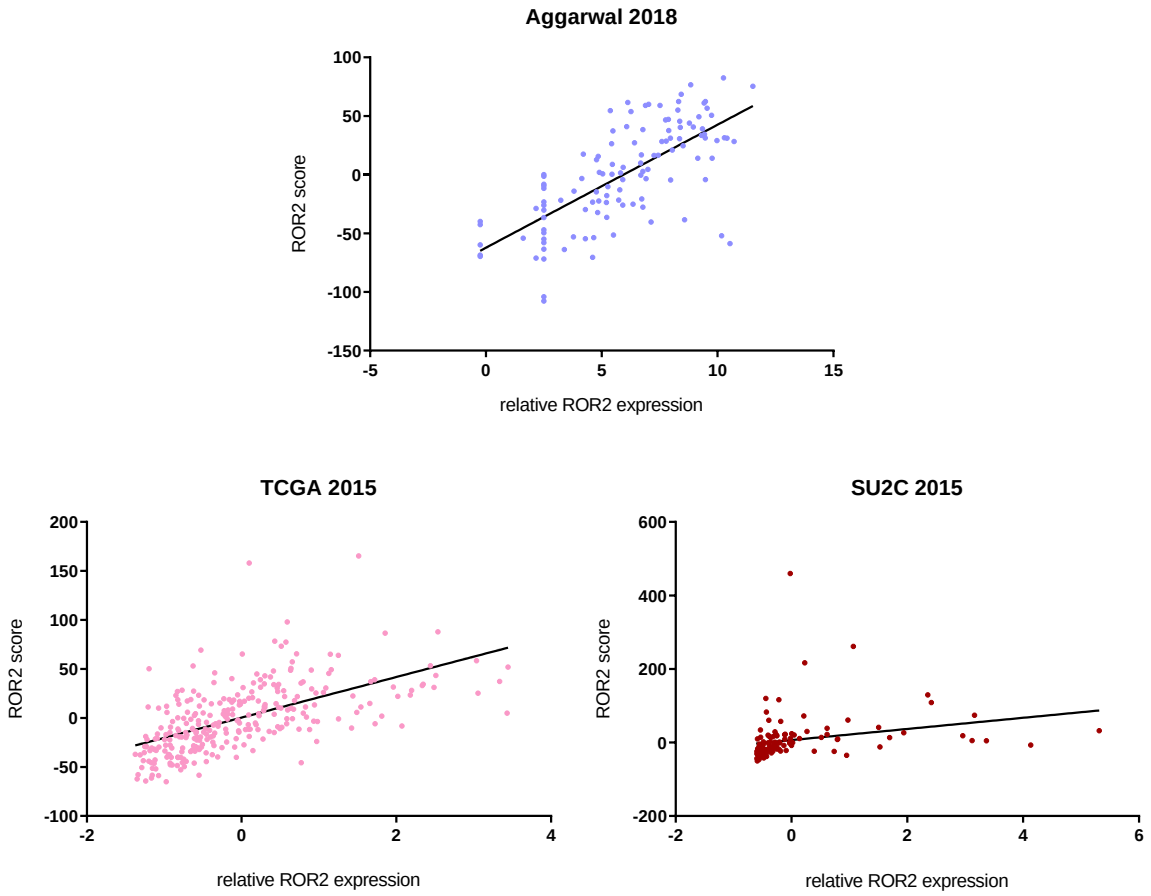


Figure 3. 2. Generated ROR2 gene signature positively correlates with ROR2 expression in various models. A) ENZ-resistant prostate cancer cell lines (42D and 42F) have elevated ROR2 expression and ROR2 gene signature score. **B)** post-castration (post-CX) mice had higher ROR2 expression and ROR2 gene signature score compared to pre-castration state (pre-CX) and full disease state (NEPC) **C)** Tumorigenic SKO and DKO GEMM mice had higher ROR2 expression and signature score compared to WT **D)** ROR2 expression and gene signature score positively correlates in three independent patient datasets.

3.3. Ligand Wnt5a Does not Affect the Expression Profile of ROR2 Gene Signature

It has been well documented that Wnt5a acts as a ligand for ROR2 in non-canonical Wnt pathway. Furthermore, the oncogenic role of Wnt5a has been elucidated in prostate cancer; Ren et al. have reported that Wnt5a induces and maintains prostate cancer dormancy in bone metastasis [111]. To investigate the role of Wnt5a in ROR2 activity in prostate cancer setting, we examined ROR2 gene signature and Wnt5a expression in two patient cohorts. Interestingly, correlation studies of ROR2 score

and Wnt5a expression indicated that Wnt5a did not have any influence on the expression profile of genes in ROR2 signature (**Figure 3.3)**. Thus, our findings showed that Wnt5a does not affect ROR2 activity and ROR2 activity maybe through ligand independent mechanism.

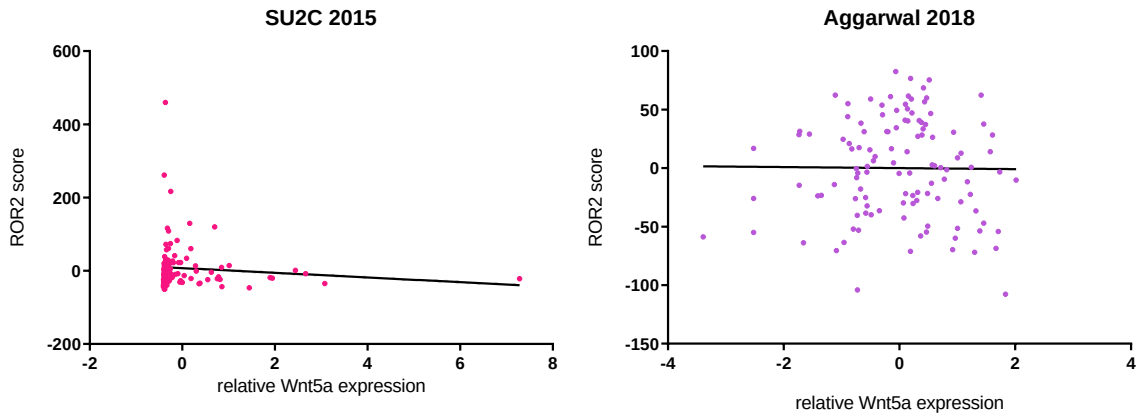


Figure 3. 3. Wnt5a expression does not affect ROR2 activity. Correlation studies of Wnt5a expression and ROR2 gene signature score in datasets of two independent patient cohorts.

3.4. Characterization of ROR2 Gene Signature in Treatment-Resistant Prostate Cancer

To gain further insight into the biology of ROR2 gene signature, we characterized the ROR2 signature and identified what molecular pathways the genes in the signature were involved in. To investigate involved molecular pathways, we utilized a program called Molecular Signatures Database (MSigDB). MSigDB uses compute overlaps (between user's gene set and a collection of annotate gene sets available in the software) to classify genes into relevant pathways. MsigDB-based analysis of ROR2 gene signature with hallmark gene sets revealed interferon-gamma (IFN-γ) response pathway as the top pathway that the genes in the ROR2 signature was involved in (**Figure 3.4)**. IFN-γ is a pleiotropic cytokine that is known to carry out diverse biological activities associated with cell-mediated adaptive immune response and immune regulation [133]. Cellular responses to IFN-γ are mediated by binding to IFN-γ receptor (IFN-γR). Binding of IFN-γ to receptor phosphorylates downstream signaling molecules (such as JAK1/2, MEK1/2) and activates transcription factors (ie. STAT1, NF-κB) [133]. In regards to cancer, the role of IFN-γ has been described as 'two faced' and exhibit both **anti-tumorigenic/immune surveillance effects** (ie.

augmentation of cytotoxicity of NK cells and cytotoxic CD8+ T cells, up-regulation of MHC class I, inhibition of tumor cell proliferation) and **pro-tumorigenic/immune evasion effects** (ie. down-regulation of tumor antigen, accumulation of myeloid-derived suppressor cells, induction of PD-L1 expression). The specific role of IFN-γ response in cancer depends on the type of tumor, microenvironment, and IFN-γ signaling intensity. Based on our observation that IFN-γ response genes were enriched with high ROR2 expression, we hypothesized that the IFN-γ response genes in generated ROR2 signature are pro-tumorigenic in nature and confer immune evasive properties to cancer cells.

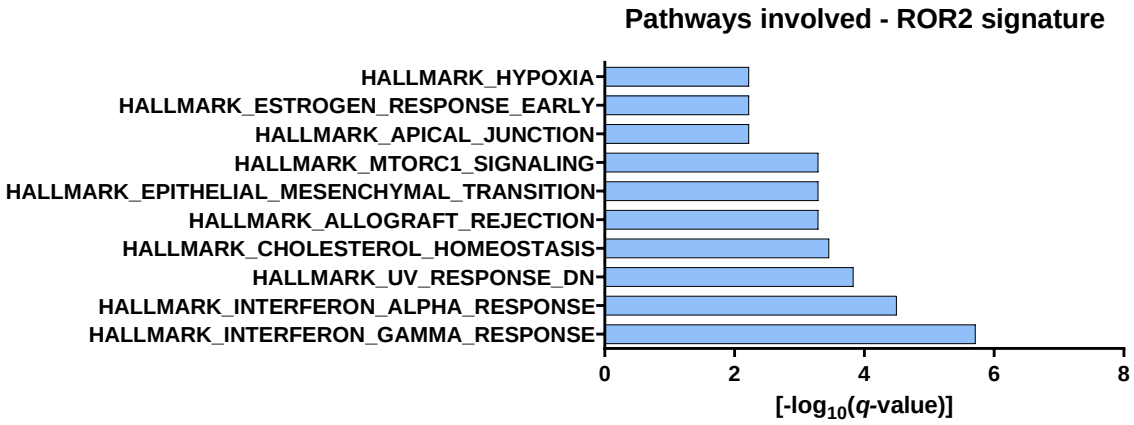


Figure 3. 4. MSigDB analysis of ROR2 gene signature. Interferon-gamma (IFN-γ) response pathway is the top pathway that the genes of the ROR2 signature is involved in.

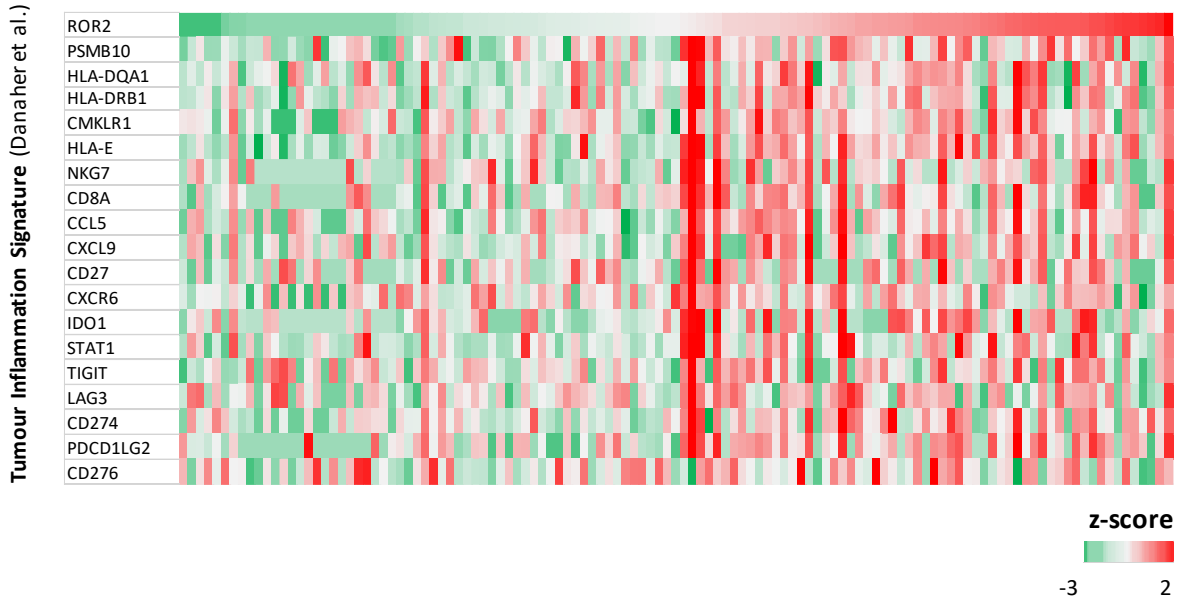
3.5. Identification of CD274 as a Potential Gene Regulated by ROR2 in Treatment-Resistant Prostate Cancer

To investigate the potential immunosuppressive effects of ROR2 in treatment-resistant prostate cancer, we utilized a published gene signature called Tumor Inflammation Signature (TIS) generated by Ayers and his colleagues [134]. TIS is an investigational use only 18-gene signature that measures a pre-existing but suppressed adaptive immune response within tumors and is enriched in patients who respond to anti-PD1 agent pembrolizumab [135]. TIS was analyzed and validated across various cancer types in over 9000 tumor expression profiles in The Cancer Genome Atlas (TCGA) [135]. We generated a heat map of TIS on patients with ENZ/ABI-resistant mCRPC (Aggarwal 2018) based on ROR2 expression (**Figure 3.5A)**. As illustrated in **Figure 3.5A**, TIS profile is positively correlated with ROR2 expression, with ROR2-high patients having elevated expression of TIS compared to ROR2-low patients. Furthermore, stable ROR2 knockdown in ENZ-resistant cell lines significantly reduced the TIS score (represented as a sum of mRNA

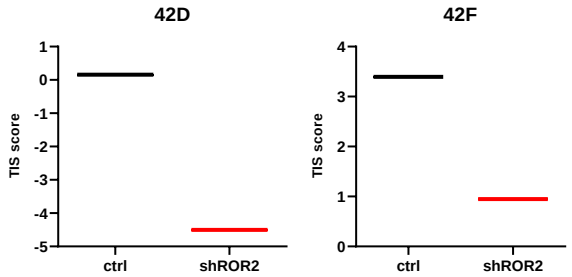
expression of genes in the signature) in ENZ-resistant CRPC cell lines (**Figure 3.5B)**. Combining these data together with MsigDB analysis of custom ROR2 gene signature strongly support that ROR2 and immune evasion are highly associated in treatment-resistant prostate cancer.

Based on our findings, we dissected the generated ROR2 gene signature and TIS to identify a potential immune-suppressive gene that is directly regulated by ROR2. We compared these two gene sets and looked for common genes present in both. Interestingly, we discovered that CD274 (programmed death-ligand 1 or PD-L1) was the only gene commonly present in both TIS and members of IFN-γ response genes in generated ROR2 gene signature (**Figure 3.5C)**. Therefore, we hypothesized that ROR2 regulates PD-L1 expression in treatment-resistant prostate cancer.

A



B



C

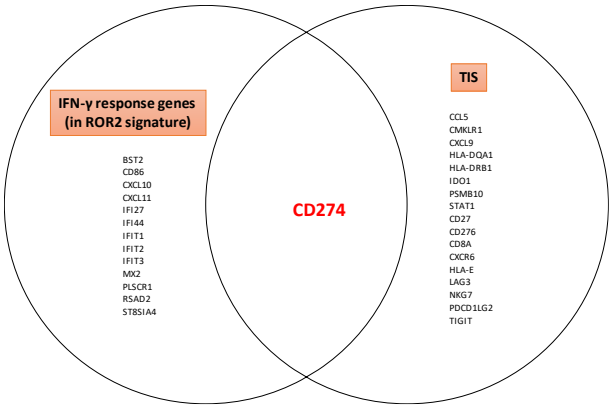
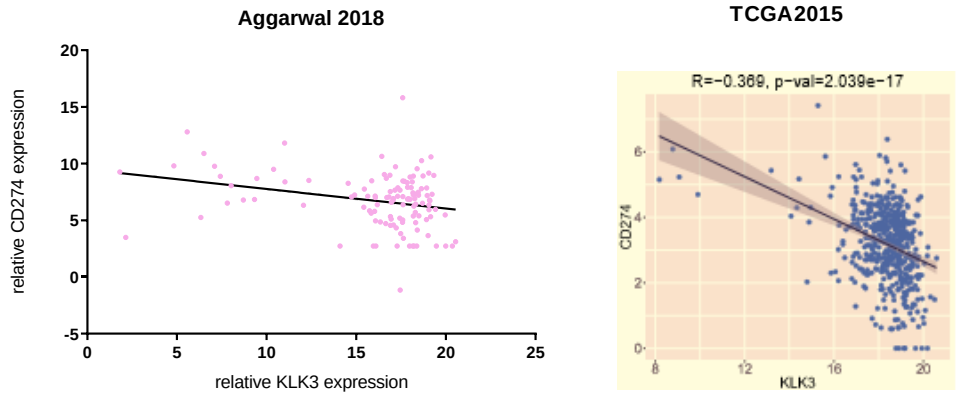


Figure 3. 5. CD274 as a potential immunosuppressive gene regulated by ROR2. A) heatmap of TIS based on ROR2 expression in data set of patients with ENZ/ABI resistant mCRPC (Aggarwal 2018) **B)** TIS scores (represented as a sum of mRNA expression) of shROR2 knockdown ENZ-resistant CRPC cell lines compared to their controls (42D and 42F) **C)** schematic diagram of identifying CD274 as the common gene between TIS and ROR2 signature

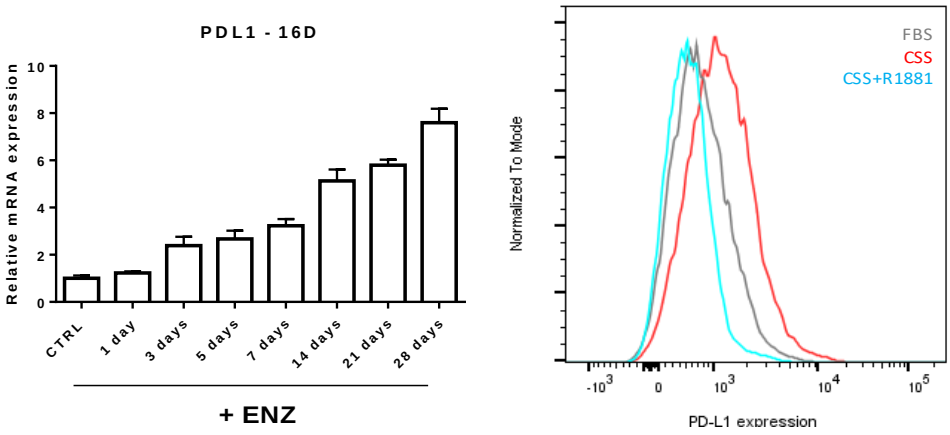
3.6. The Effect of AR Activity on ROR2 and CD274 Expression

Androgen receptor and its activity plays a central role in pathogenesis of prostate cancer. It is previously reported by Fankhauser and his colleagues that PD-L1 expression is scarce in prostate adenocarcinoma and CRPC, but is highly upregulated in ENZ-resistant prostate cancer [136]. However, it is still unclear how the ENZ resistance confers the PD-L1 upregulation in treatment-resistant prostate cancer. Since ENZ inhibits the activity of AR, we hypothesized that ENZ-dependent suppression of AR activity drives the over-expression of PD-L1. To further investigate the role of AR activity in PD-L1 expression, we performed correlation studies on expression of CD274 and one of major indicators of AR activity, KLK3 (prostate specific antigen, PSA) in two patient cohorts. As illustrated in **Figure 3.6A**, correlation studies of patient data showed negative correlation between CD274 and AR activity. Furthermore, androgen deprivation condition drives PD-L1 upregulation and restoring AR activity through AR agonist (R1881) restored low-PD-L1 phenotype in CRPC cell line (**Figure 3.6B)**. Similarly, we investigated the role of AR activity in ROR2 expression. From our previous findings (**Figure 1.8**), we saw an increased ROR2 expression in ENZ-resistant prostate cancer model. Since we identified CD274 as a potential downstream target for ROR2, we expected to see a similar pattern in ROR2-AR as we did in CD274-AR. Through correlation studies, we found that ROR2 expression is negatively correlated with AR activity (**Figure 3.6C)**. Correlation studies of ROR2 signature score and KLK3 expression and AR score and ROR2 expression further demonstrated the inverse relationship between ROR2 and AR (**Figure 3.6D and 3.6E)**. In vitro experiments validated the observation; androgen deprivation in CRPC cell line 16D resulted in upregulation of ROR2, both at mRNA and protein level (**Figure 3.6F).** Our findings demonstrated AR suppression of both CD274 and ROR2 and androgen deprivation resulted in upregulation of both genes.

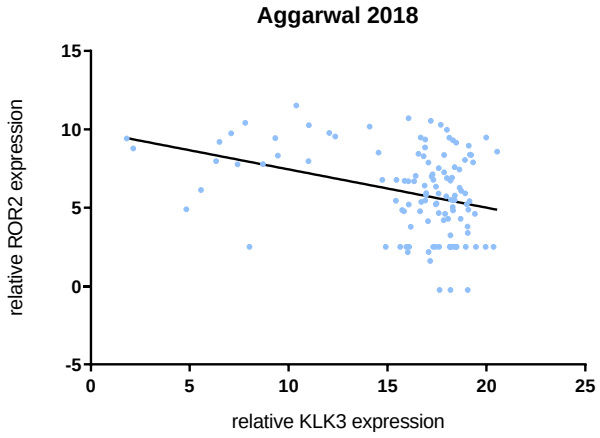
A


Aggarwal 2018
relative CD274 expression
relative KLK3 expression
TCGA2015
R=-0.369, p-val=2.039e-17
CD274
KLK3

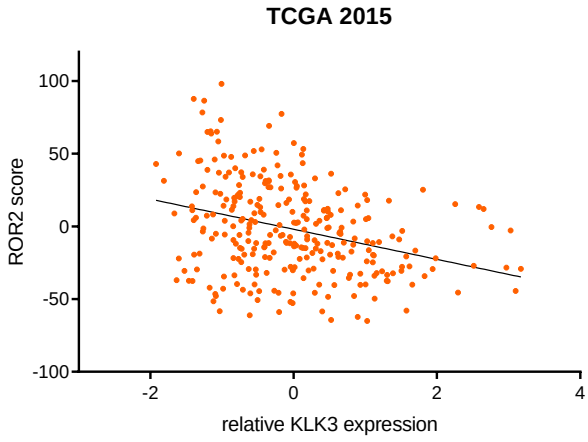
B


PDL1 - 16D
Relative mRNA expression
CTRL
1 day
3 days
5 days
7 days
14 days
21 days
28 days
+ ENZ
FBS
CSS
CSS+R1881
Normalized To Mode
PD-L1 expression

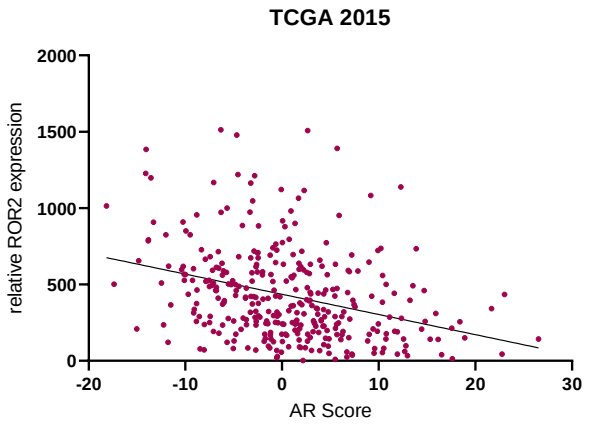
C


Aggarwal 2018
relative ROR2 expression
relative KLK3 expression

D


TCGA 2015
ROR2 score
relative KLK3 expression

E



F

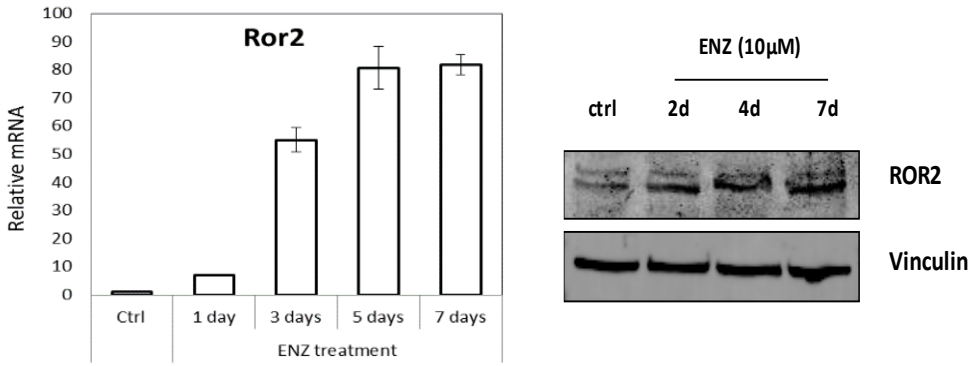
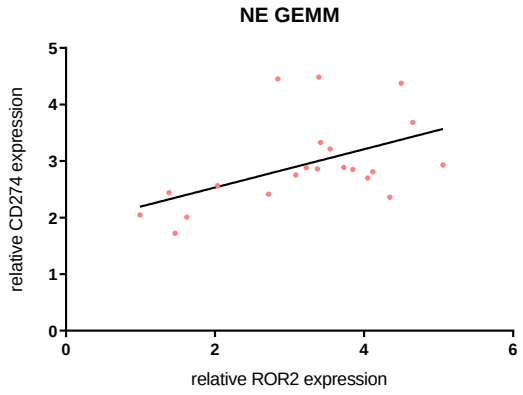


Figure 3. 6. AR suppresses both CD274 and ROR2. A) correlation studies of CD274 and KLK3 in two independent patient cohorts (Aggarwal and TCGA) **B)** mRNA (**left**) and protein (**right**) expression of CD274 in 16D under androgen deprivation conditions **C)** correlation studies of ROR2 and KLK3 in two independent patient cohorts (Aggarwal and TCGA) **D)** correlation study of ROR2 signature and KLK3 expression in a patient cohort (TCGA) **E)** correlation study of AR score and ROR2 expression in a patient cohort (TCGA) **F)** mRNA (**left**) and protein (**right**) expression of ROR2 in 16D under androgen deprivation condition induced by ENZ.

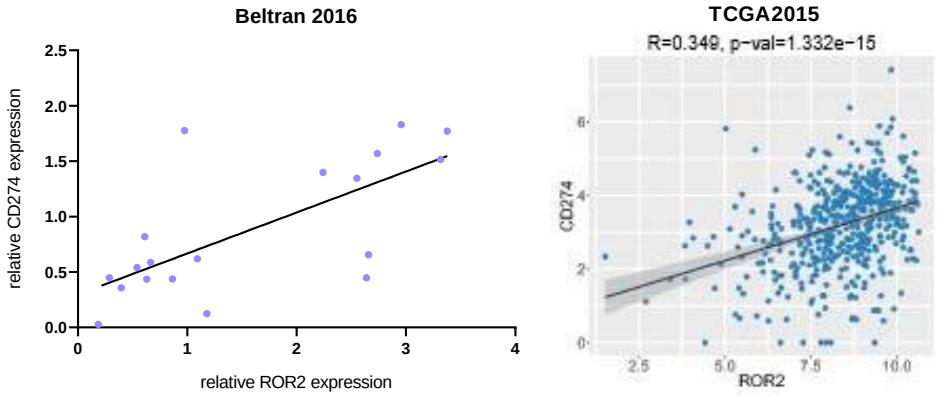
3.7. Expression of ROR2 and CD274 are Highly Correlated

In an effort to further examine a potential ROR2-CD274 axis, we looked at the expression profiles of ROR2 and CD274 in various data sets. As expected, we found that ROR2 and CD274 are positively correlated in both GEMM mice model (**Figure 3.7A)** and patient cohorts (**Figure 3.7B**). This observation was not limited to just prostate cancer but also present in other cancer types (**Figure 3.7C)**. Based on the observation of positive correlation between ROR2 and CD274, we wanted to investigate whether CD274 and ROR2 were co-expressed. The volcano plot of GEMM mice model (between DKO and WT) revealed co-enrichment of ROR2 and CD274 in DKO **(Figure 3.7D)**. Both ROR2 signature and TIS are enriched in the tumorigenic DKO compared to WT. Furthermore, this observation was consistent in patients; CD274 was enriched in $ROR2_{high}$ population (**Figure 3.7E)**. Interestingly, the expression plot of Aggarwal dataset showed high co-expression of ROR2 and CD274 regardless of their clinical subtypes (small cell prostate cancer (SCPC) and non-SCPC), supporting our phenotypic observation in in vitro models (**Figure 3.7F**).

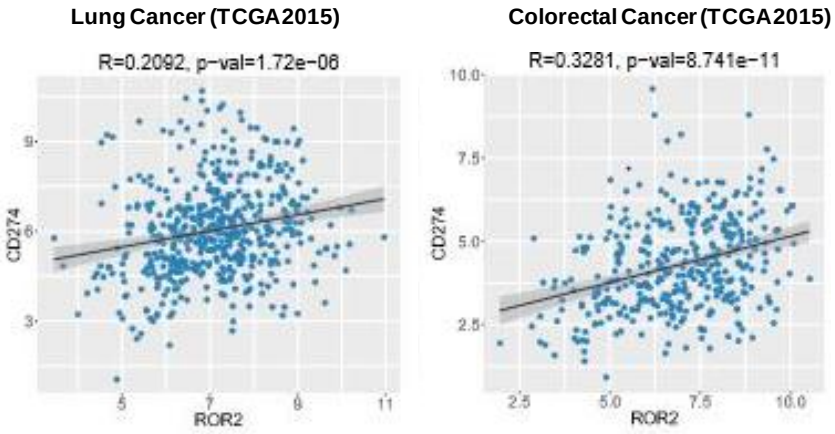
A


NE GEMM
relative CD274 expression
relative ROR2 expression
0
1
2
3
4
5
0
2
4
6

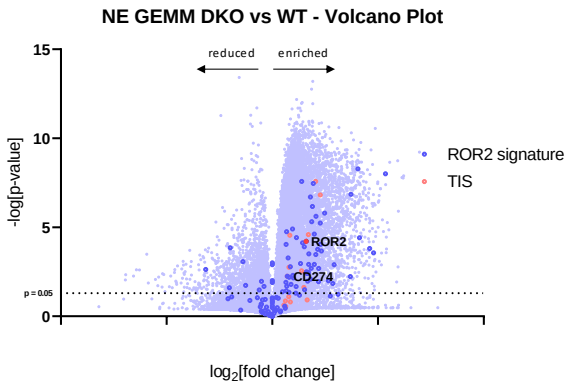
B


Beltran 2016
relative CD274 expression
relative ROR2 expression
0.0
0.5
1.0
1.5
2.0
2.5
0
1
2
3
4
TCGA2015
R=0.349, p-val=1.332e-15
CD274
ROR2
0
2
4
6
2.5
5.0
7.5
10.0

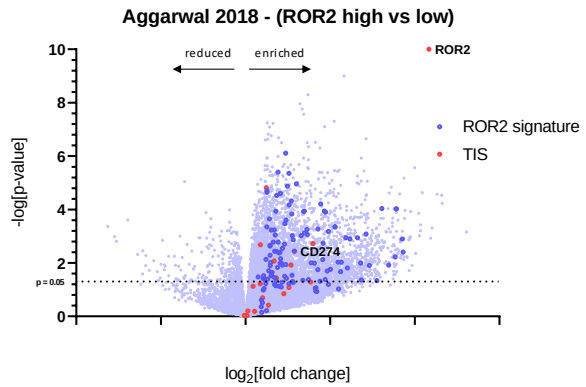
C


Lung Cancer (TCGA2015)
R=0.2092, p-val=1.72e-06
CD274
ROR2
3
6
9
5
7
9
11
Colorectal Cancer (TCGA2015)
R=0.3281, p-val=8.741e-11
CD274
ROR2
2.5
5.0
7.5
10.0
2.5
5.0
7.5
10.0

D



E



F

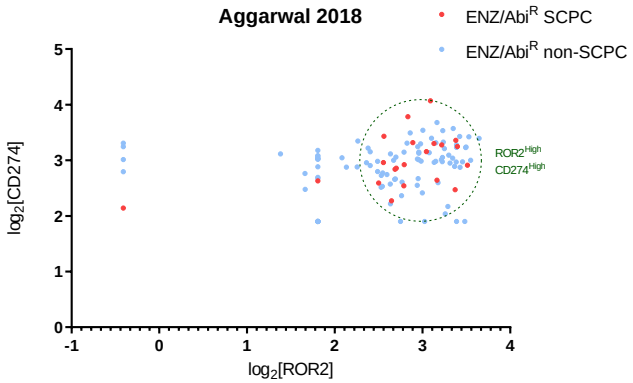
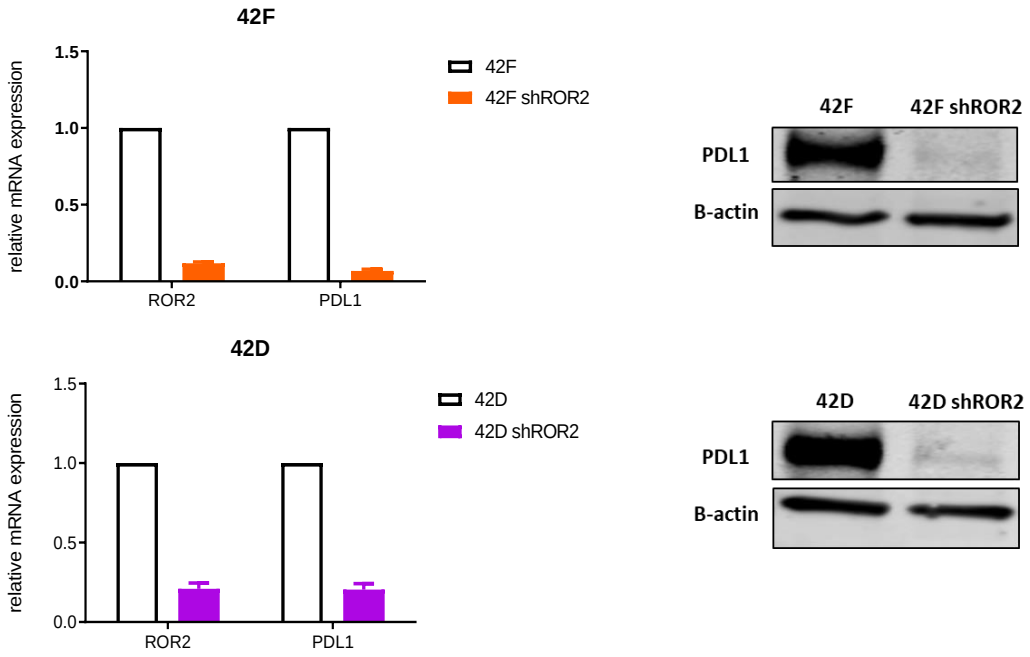


Figure 3. 7. Expression of ROR2 and CD274 are highly correlated and co-expressed. A) correlation study of CD274 and ROR2 in NE GEMM mice model **B)** correlation studies of CD274 and ROR2 in two independent patient cohorts (Beltran and TCGA) **C)** correlation studies of CD274 and ROR2 in lung cancer and colorectal cancer patients (TCGA) **D)** volcano plot of enriched and reduced genes in DKO compared to WT in NE GEMM dataset **E)** volcano plot of enriched and reduced genes in $ROR2_{high}$ compared to $ROR2_{low}$ in Aggarwal patient dataset **F)** Expression plot of CD274 and ROR2 in Aggarwal patient dataset

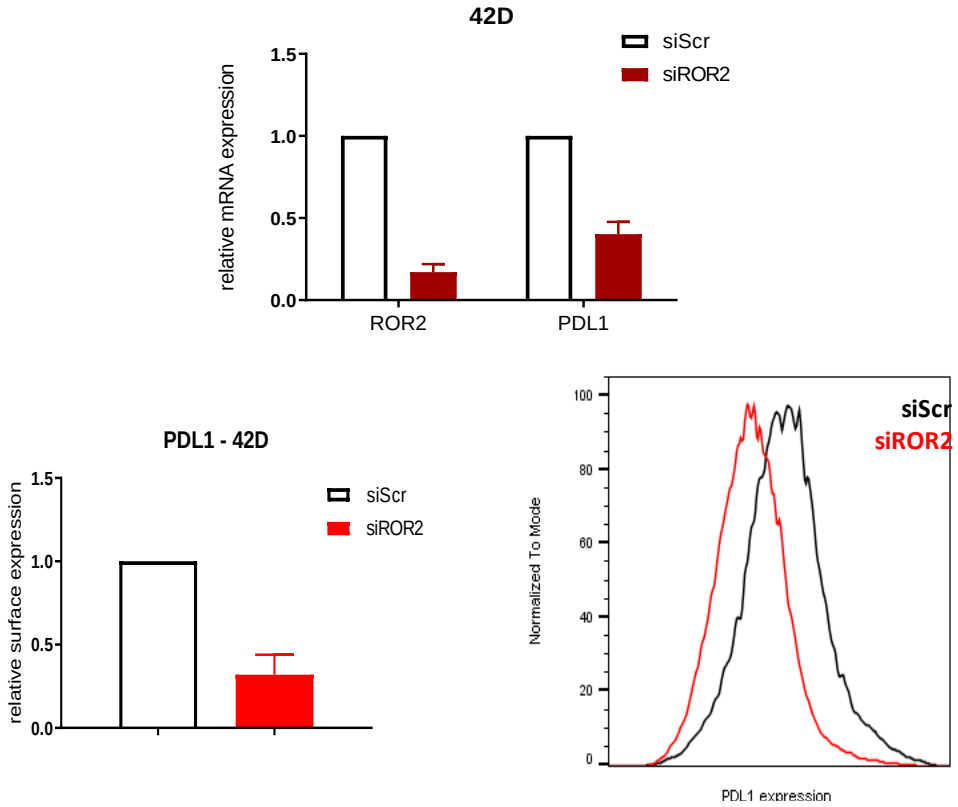
3.8. In Vitro Validation of ROR2-CD274 Axis in Treatment-Resistant Prostate Cancer

To demonstrate that ROR2 is required to induce CD274 expression in treatment-resistant prostate cancer, we modulated the expression of ROR2 in in vitro cell lines and assessed the expression of CD274. First, we looked at established stable ROR2 knockdown cell lines (42D shROR2 and 42F shROR2) and examined PD-L1 expression. Stable knockdown of ROR2 significantly downregulated PD-L1 expression at both mRNA and protein level (**Figure 3.8A**). This was consistent in transient knockdown of ROR2 with siRNA (**Figure 3.8B**). Next, we examined whether the overexpression of ROR2 was sufficient to drive PD-L1 upregulation in parental CRPC cell line (16D). As illustrated in **Figure 3.8C**, overexpression upregulated PD-L1 expression. Lastly, we wanted to examine whether targeting ROR2 was sufficient to prevent ENZ-driven upregulation of PD-L1. When we co-treated 16D with siROR2 and ENZ, it successfully prevented the upregulation of PD-L1 (**Figure 3.8D**). Therefore, our in vitro experiments validated our previous findings.

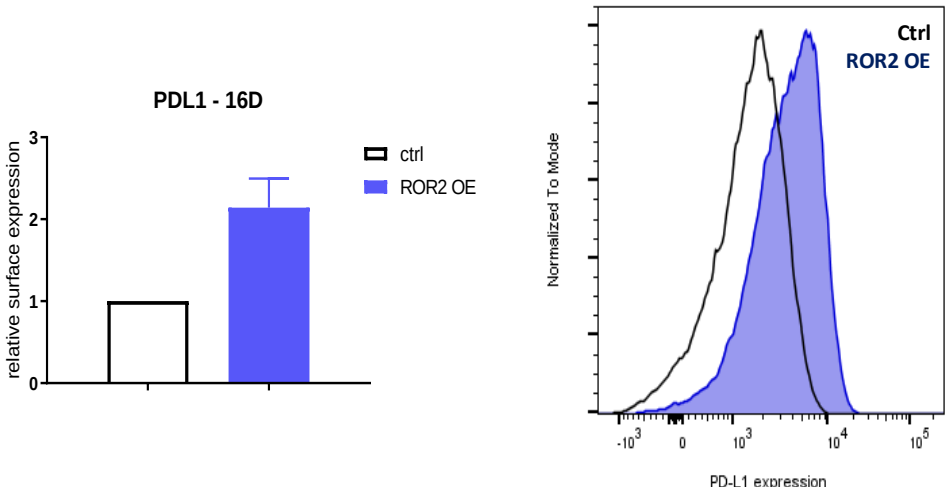
A


42F
relative mRNA expression
1.5
1.0
0.5
0.0
ROR2
PDL1
42F
42F shROR2
42F
42F shROR2
PDL1
B-actin
42D
relative mRNA expression
1.5
1.0
0.5
0.0
ROR2
PDL1
42D
42D shROR2
42D
42D shROR2
PDL1
B-actin

B


42D
relative mRNA expression
1.5
1.0
0.5
0.0
ROR2
PDL1
siScr
siROR2
PDL1 - 42D
relative surface expression
1.5
1.0
0.5
0.0
siScr
siROR2
Normalized To Mode
100
80
60
40
20
0
siScr
siROR2
PDL1 expression

C



D

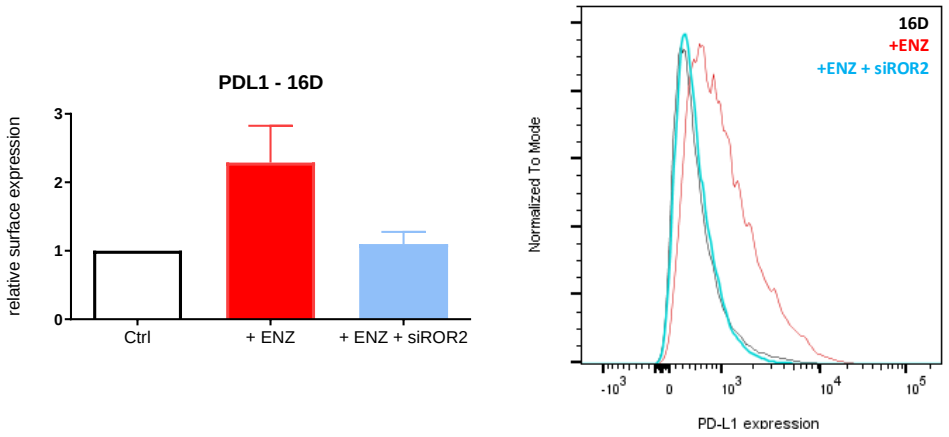


Figure 3. 8. In vitro experiments confirm the ROR2-PDL1 axis in ENZ-resistant prostate cancer. A) mRNA (left) and protein (right) expression of ROR2 and PDL1 in shROR2 stable cell lines (42D and 42F) compared to their controls **B)** mRNA expression (top) and protein (bottom) expression of PDL1 in siROR2 treated ENZ-resistant cell lines. Protein expression was measured by flow cytometry. **C)** protein expression of PDL1 in ROR2 overexpressed 16D compared to control, measured by flow cytometry. **D)** protein expression of PDL1 in 16D; control, treated with 10µM ENZ (7 days), and co-treated with ENZ and siROR2 (7 days). Protein expression was measured by flow cytometry.

4. Discussion

Although there has been a vast improvement in developing potent antiandrogens, treatment resistance is an inevitable fate for patients with advanced form of prostate cancer. Therefore, it is crucial to understand the molecular mechanism of treatment resistance in progression of prostate cancer to identify novel therapeutic targets, provide rationale for combination therapy, and discover biomarkers. To date, effort to understand the resistance mechanism has only been made partially with much of the known findings focusing on the androgen pathway and it is still unclear what other molecular events occur that provide biological advantage for cancer cells. Since dysregulation of various RTKs have shown to possess oncogenic properties in various cancers, we wanted to focus on the role of RTKs in treatment resistance in prostate cancer.

To identify a potential RTK with an oncogenic role in treatment-resistant prostate cancer, we investigated the expression of all known human RTKs in in vitro cell lines and a PDX mouse model. As a result, we identified ROR2 as one of the top RTKs upregulated in treatment resistance. Moreover, this observation was supported by clinical evidence, establishing ROR2 as the only RTK upregulated in both in vitro models and patients. Previous reports have elucidated the dual role of ROR2 as either a tumor suppressor or an oncogene, and dysregulation of ROR2 have been reported in various cancer types [105]. In prostate cancer, other groups showed that ROR2 promotes cell migration and invasion [108, 109]. However, it is still unclear what the role of ROR2 is in context of treatment resistance and advanced cancer. Based on our preliminary findings, we hypothesized that ROR2 plays a role in treatment-resistant prostate cancer.

In order to eliminate any bias associated with the process of characterizing the role of ROR2, we generated a novel ROR2 gene signature using RNA sequencing data of stable ROR2 knockdown cell lines (42D shROR2 and 42F shROR2) and a patient dataset. The clinical dataset we used were from Aggarwal and his colleagues and it represented our treatment-resistant prostate cancer models well due to their inclusion of patients that had progressive metastatic CRPC despite being on treatment (ENZ or ABI). Generated ROR2 signature consisted of a list of genes that correlated with the ROR2 expression in all three independent data sets. Similar to our observation, positive correlations between the ROR2 expression and gene signature were observed in other data sets (including mouse models and patient cohorts) and further validated the legitimacy of the signature. Here, we are the first to develop a novel gene signature for ROR2 in which can provide insights on ROR2 activity based on expression patterns. We believe that ROR2 gene signature can conveniently be utilized to gather primary baseline on ROR2 signaling before molecularly

dissecting the downstream signaling cascades. Further supporting the rationale of using RTK-based gene signature, various studies have reported the usage of EGFR-based gene signature in different cancer types [137-139]. In a future study, generating a phosphorylation-based microarray profile for ROR2 could be a useful tool that can be used in conjunction with ROR2 gene signature. Also, investigation of ROR2 signature based on clinical attributes (such as overall survival) could be used to determine the suitability of ROR2 as a prognostic marker.

Wnt5a is involved in activating several non-canonical Wnt signaling pathways and is a known ligand for ROR2 [140]. The oncogenic properties of Wnt5a has been elucidated in various cancer types such as melanoma, although the exact role it plays does not completely overlap with ROR2 due to its multi-receptor binding nature [108,109,127]. Since ROR2 signaling normally depends on ligand binding like other conventional RTKs, we expected Wnt5a to have significant influence to ROR2 activity. Interestingly, our findings showed that Wnt5a has no correlation with ROR2 gene signature and therefore does not affect ROR2 activity in treatment-resistant prostate cancer. Based on our observation, we believe that ROR2 can be constitutively activated in a ligand-independent manner, which have been observed with other RTKs such as EGFR and c-kit in various cancers [141, 142]. For example, EGFR is reported to be constitutively activated without its ligand in glioblastoma and ligand-independent activation of EGFR signals through a non-canonical IRF3 signaling pathway that is mutually exclusive from ligand-dependent EGFR signaling cascade [129]. Similar to EGFR, Wnt5a-dependent and Wnt5a-independent activation of ROR2 could both exist and downstream signaling cascades could be distinct from each other. Furthermore, due to its versatility to bind to different receptors, Wnt5a signaling observed in prostate cancer could depend on other receptors such as FZD. All combined, it provided a partial explanation to the potential mutual exclusivity in oncogenic roles of Wnt5a and ROR2 and a necessary rationale to further characterize the ROR2 signature to elucidate a novel functionality of ROR2. In a future study, in vitro experiments involving Wnt5a are required to fully validate the ligand-independence of ROR2 activity; for example, Wnt5a agonists can be used and the resulting phenotype can be analyzed.

In order to characterize ROR2 signature, we classified the genes in the signature into reported molecular pathways and ranked them using MSigDB software. As a result, we found that interferon-gamma (IFN-γ) response pathway was the top molecular pathway that genes of ROR2 signature was involved in. IFN-γ is a well-known cytokine that carries out diverse functions in adaptive immune system and cellular responses to IFN-γ have been described to have both pro- and anti- tumorigenic effects [123]. Given the fact that upregulation of ROR2 expression and signature occurred in treatment resistance, we

believed that ROR2-dependent activation of IFN-γ response pathway was pro-tumorigenic in nature and conferred immunosuppressive properties to cancer cells. To further investigate the potential link between ROR2 and immune evasion, we looked at the expression profiles of known immune suppression markers (TIS) and ROR2 in patients. We found that ROR2 expression was highly correlative with TIS. This observation was further supported in in vitro models where we found that TIS expression was significantly reduced with knockdown of ROR2. Investigating TIS and IFN-γ response pathway revealed that CD274 (PD-L1) was the only gene present in both gene sets. The role of CD274 in conferring immune evasive properties to cancer cells have been documented in many cancers and is one of the main targets for immunotherapy. In prostate cancer, our laboratory has previously reported that the expression of CD274 significantly increased in Enzalutamide resistant prostate cancer [119]. However, it is still unknown through what molecular mechanism the CD274 upregulation occurs. This finding suggests for the first time that there may be a link between ROR2 and CD274.

It is well known that androgen receptor and its activity are central to prostate cancer [13]. Therefore, we decided to investigate the relationship of ROR2 and CD274 with AR to provide insights on a potential ROR2-CD274 axis. Given that upregulation of ROR2 and CD274 occur in androgen deprived condition, we expected that both ROR2 and CD274 behave in similar ways to AR. As expected, correlation studies in patient datasets revealed that both ROR2 and CD274 are negatively correlated to AR activity. Furthermore, AR-driven suppression of ROR2 and CD274 was confirmed in in vitro cell line models. From our findings, we report that both ROR2 and CD274 are AR suppressed genes.

Moreover, evidence of ROR2-CD274 axis was further provided by series of correlation studies and co-enrichment studies in various datasets. Here, we report that ROR2 and CD274 expressions are positively correlated in a mouse model (NE-GEMM) and patient cohorts (Beltran 2016 & TCGA 2015). Interestingly, this finding was consistent across other cancer types (lung cancer and colorectal cancer), suggesting existence of ROR2-CD274 axis in a “pan-cancer” manner. These findings were supported by series of co-enrichment studies which revealed upregulation of CD274 and ROR2 in the same population. All combined, our findings provided strong rationale to the existence of ROR2-CD274 axis in prostate cancer.

Most importantly, we successfully demonstrated and validated the regulatory role of ROR2 in CD274 expression in various in vitro models. We showed that ROR2 knockdown significantly reduces the expression of CD274 in ENZ-resistant CRPC cell lines. Also, overexpression of ROR2 in ENZ-sensitive CRPC cell line significantly induced the expression of CD274. Moreover, we showed that targeting ROR2 was

sufficient in preventing ENZ-dependent upregulation of CD274. These in vitro findings show that ROR2 is upstream of CD274 and the reported upregulation of CD274 is through ROR2 in treatment-resistance prostate cancer. Our study is the first to describe a novel role of ROR2 in immune suppression and whom identified ROR2-CD274 axis.

In summary, our study reveals a novel role of ROR2 in regulating CD274 expression in treatment-resistant prostate cancer. For the first time, we generated a custom ROR2 gene signature that tightly coordinates ROR2 expression with ROR2 activity. Through our ROR2 gene signature, we are able to provide insights on potential Wnt5a-independent activation of ROR2, as well as characterizing the function of ROR2 in treatment-resistant prostate cancer. Specifically, we identify CD274 as a gene of interest and through combination of in vitro experiments and various computational studies, we are able to legitimize the existence of ROR2-CD274 axis. Our findings provide a necessary explanation to the mechanism of ENZ-driven upregulation of CD274 reported in the literature [119]. Limitation of this study is the lack of in vivo validation of ROR2-CD274 axis, mainly due to shortage of time.

In the future, identification of a transcription factor that acts as a 'bridge' between ROR2 and CD274 is essential in understanding the exact molecular mechanism of ROR2-CD274 axis. There are significant overlaps between downstream signaling pathways of ROR2 and upstream regulatory pathways of CD274 expression, resulting in the presence of various transcription factors common in both axes. It is reported that CD274 expression is regulated by various transcription factors including STAT1, STAT3, NF-κB, IRF1, BRD4, and HIF1a [143]. Thus, identifying a specific transcription factor is required to map out the molecular pathway between ROR2 and CD274. Furthermore, in vitro experiments are necessary to validate a potential ligand-independent activity of ROR2 in treatment-resistant prostate cancer. Other RTKs have shown the capacity to be activated without their respective ligands and it may well be the case for ROR2. If validated, the exact mechanism of how ROR2 get activated poses another question to be investigated. Lastly, the exact role of CD274 in treatment-resistant prostate cancer still needs to be answered. Conventionally, CD274 acts as an immune suppressive checkpoint molecule. However, alternative functions of CD274 have been proposed in the literature. For example, there have been evidence for CD274 to act as a pro-survival signaling molecule in cancer cells in the absence of T cells [92]. Validation of the exact role of PD-L1 in prostate cancer context could be useful in understanding the mechanism of treatment resistance.

Bibliography

1. Wilson, A.H., *The prostate gland: a review of its anatomy, pathology, and treatment.* JAMA, 2014. **312**(5): p. 562.
2. Bhavsar, A. and S. Verma, *Anatomic imaging of the prostate.* Biomed Res Int, 2014. **2014**: p. 728539.
3. Kumar, V.L. and P.K. Majumder, *Prostate gland: structure, functions and regulation.* Int Urol Nephrol, 1995. **27**(3): p. 231-43.
4. McNeal, J.E., *The zonal anatomy of the prostate.* Prostate, 1981. **2**(1): p. 35-49.
5. Walsh, P.C., Retik, A. B., Stamey, T.A., Vaughan, E.D.Jr. , *Campbell's Urology.* . W.B. Saunders Co., 1992.
6. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
7. Taitt, H.E., *Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location.* Am J Mens Health, 2018. **12**(6): p. 1807-1823.
8. Chan, J.M., P.H. Gann, and E.L. Giovannucci, *Role of diet in prostate cancer development and progression.* J Clin Oncol, 2005. **23**(32): p. 8152-60.
9. van Breemen, R.B. and N. Pajkovic, *Multitargeted therapy of cancer by lycopene.* Cancer Lett, 2008. **269**(2): p. 339-51.
10. Chen, H., et al., *RNASEL mutations in hereditary prostate cancer.* J Med Genet, 2003. **40**(3): p. e21.
11. Yin, M., et al., *Prevalence of incidental prostate cancer in the general population: a study of healthy organ donors.* J Urol, 2008. **179**(3): p. 892-5; discussion 895.
12. Etzioni, R., et al., *Impact of PSA screening on the incidence of advanced stage prostate cancer in the United States: a surveillance modeling approach.* Med Decis Making, 2008. **28**(3): p. 323-31.
13. Salami, S.S., et al., *Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer.* Urol Oncol, 2013. **31**(5): p. 566-71.
14. Chen, N. and Q. Zhou, *The evolving Gleason grading system.* Chin J Cancer Res, 2016. **28**(1): p. 58-64.
15. Gordetsky, J. and J. Epstein, *Grading of prostatic adenocarcinoma: current state and prognostic implications.* Diagnostic Pathology, 2016. **11**.
16. Danneman, D., et al., *Gleason inflation 1998-2011: a registry study of 97,168 men.* BJU Int, 2015. **115**(2): p. 248-55.
17. Knudsen, B.S. and V. Vasioukhin, *Mechanisms of prostate cancer initiation and progression.* Adv Cancer Res, 2010. **109**: p. 1-50.
18. Kristal, A.R., et al., *Androgen receptor CAG repeat length is not associated with the risk of incident symptomatic benign prostatic hyperplasia: results from the Prostate Cancer Prevention Trial.* Prostate, 2010. **70**(6): p. 584-90.
19. Brawer, M.K., *Prostatic intraepithelial neoplasia: an overview.* Rev Urol, 2005. **7 Suppl 3**: p. S11-8.
20. Cheville, J.C., M.J. Reznicek, and D.G. Bostwick, *The focus of "atypical glands, suspicious for malignancy" in prostatic needle biopsy specimens: incidence, histologic features, and clinical follow-up of cases diagnosed in a community practice.* Am J Clin Pathol, 1997. **108**(6): p. 633-40.
21. Ayala, A.G. and J.Y. Ro, *Prostatic intraepithelial neoplasia: recent advances.* Arch Pathol Lab Med, 2007. **131**(8): p. 1257-66.
22. Nelson, W.G., A.M. De Marzo, and S. Yegnasubramanian, *Epigenetic Alterations in Human Prostate Cancers.* Endocrinology, 2009. **150**(9): p. 3991-4002.

23. Enokida, H., et al., *Multigene methylation analysis for detection and staging of prostate cancer.* Clinical Cancer Research, 2005. **11**(18): p. 6582-6588.
24. Helgeson, B.E., et al., *Characterization of TMPRSS2 : ETV5 and SLC45A3 : ETV5 gene fusions in prostate cancer.* Cancer Research, 2008. **68**(1): p. 73-80.
25. Tomlins, S.A., et al., *The role of SPINK1 in ETS rearrangement-negative prostate cancers.* Cancer Cell, 2008. **13**(6): p. 519-528.
26. Ashida, S., et al., *Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs.* Cancer Res, 2004. **64**(17): p. 5963-72.
27. Heinlein, C.A. and C. Chang, *Androgen receptor in prostate cancer.* Endocr Rev, 2004. **25**(2): p. 276-308.
28. Lamb, A., Neal, D., *Role of the androgen receptor in prostate cancer.* Trends in Urology & Men's Health, 2013: p. 26-30.
29. Huggins, C. and C.V. Hodges, *Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate.* CA Cancer J Clin, 1972. **22**(4): p. 232-40.
30. Tan, M.H., et al., *Androgen receptor: structure, role in prostate cancer and drug discovery.* Acta Pharmacol Sin, 2015. **36**(1): p. 3-23.
31. Filson, C.P., L.S. Marks, and M.S. Litwin, *Expectant management for men with early stage prostate cancer.* CA Cancer J Clin, 2015. **65**(4): p. 265-82.
32. Walsh, P.C., *Radical prostatectomy for localized prostate cancer provides durable cancer control with excellent quality of life: a structured debate.* J Urol, 2000. **163**(6): p. 1802-7.
33. Crawford, E.D., et al., *Androgen-targeted therapy in men with prostate cancer: evolving practice and future considerations.* Prostate Cancer Prostatic Dis, 2019. **22**(1): p. 24-38.
34. Ortmann, O., J.M. Weiss, and K. Diedrich, *Gonadotrophin-releasing hormone (GnRH) and GnRH agonists: mechanisms of action.* Reprod Biomed Online, 2002. **5 Suppl 1**: p. 1-7.
35. Sharifi, N., J.L. Gulley, and W.L. Dahut, *Androgen deprivation therapy for prostate cancer.* JAMA, 2005. **294**(2): p. 238-44.
36. Aragon-Ching, J.B. and W.L. Dahut, *Novel Androgen Deprivation Therapy (ADT) in the Treatment of Advanced Prostate Cancer.* Drug Discov Today Ther Strateg, 2010. **7**(1-2): p. 31-35.
37. Chandrasekar, T., et al., *Mechanisms of resistance in castration-resistant prostate cancer (CRPC).* Transl Androl Urol, 2015. **4**(3): p. 365-80.
38. Litwin, M.S. and H.J. Tan, *The Diagnosis and Treatment of Prostate Cancer: A Review.* JAMA, 2017. **317**(24): p. 2532-2542.
39. Schalken, J. and J.M. Fitzpatrick, *Enzalutamide: targeting the androgen signalling pathway in metastatic castration-resistant prostate cancer.* BJU Int, 2016. **117**(2): p. 215-25.
40. Sumanasuriya, S. and J. De Bono, *Treatment of Advanced Prostate Cancer-A Review of Current Therapies and Future Promise.* Cold Spring Harb Perspect Med, 2018. **8**(6).
41. Gschwind, A., O.M. Fischer, and A. Ullrich, *The discovery of receptor tyrosine kinases: targets for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 361-70.
42. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases.* Cell, 2010. **141**(7): p. 1117-34.
43. Carraway, K.L., 3rd and L.C. Cantley, *A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling.* Cell, 1994. **78**(1): p. 5-8.
44. Lemmon, M.A. and J. Schlessinger, *Regulation of signal transduction and signal diversity by receptor oligomerization.* Trends Biochem Sci, 1994. **19**(11): p. 459-63.
45. Olayioye, M.A., et al., *The ErbB signaling network: receptor heterodimerization in development and cancer.* EMBO J, 2000. **19**(13): p. 3159-67.

46. Nolen, B., S. Taylor, and G. Ghosh, *Regulation of protein kinases; controlling activity through activation segment conformation.* Mol Cell, 2004. **15**(5): p. 661-75.
47. Cobb, M.H., et al., *Autophosphorylation activates the soluble cytoplasmic domain of the insulin receptor in an intermolecular reaction.* J Biol Chem, 1989. **264**(31): p. 18701-6.
48. Till, J.H., et al., *Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation.* Structure, 2002. **10**(9): p. 1187-96.
49. Favelyukis, S., et al., *Structure and autoregulation of the insulin-like growth factor 1 receptor kinase.* Nat Struct Biol, 2001. **8**(12): p. 1058-63.
50. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.
51. Du, Z. and C.M. Lovly, *Mechanisms of receptor tyrosine kinase activation in cancer.* Mol Cancer, 2018. **17**(1): p. 58.
52. Brummer, T., C. Schmitz-Peiffer, and R.J. Daly, *Docking proteins.* FEBS J, 2010. **277**(21): p. 4356-69.
53. Ingham, R.J., et al., *The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor.* J Biol Chem, 1998. **273**(46): p. 30630-7.
54. Sun, T., et al., *Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase.* Cell, 2011. **144**(5): p. 703-18.
55. Ludes-Meyers, J.H., et al., *Transcriptional activation of the human epidermal growth factor receptor promoter by human p53.* Mol Cell Biol, 1996. **16**(11): p. 6009-19.
56. Reznik, T.E., et al., *Transcription-dependent epidermal growth factor receptor activation by hepatocyte growth factor.* Mol Cancer Res, 2008. **6**(1): p. 139-50.
57. Rodriguez-Antona, C., et al., *Overexpression and activation of EGFR and VEGFR2 in medullary thyroid carcinomas is related to metastasis.* Endocr Relat Cancer, 2010. **17**(1): p. 7-16.
58. Hanawa, M., et al., *EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus.* Int J Cancer, 2006. **118**(5): p. 1173-80.
59. Lopez-Gines, C., et al., *New pattern of EGFR amplification in glioblastoma and the relationship of gene copy number with gene expression profile.* Mod Pathol, 2010. **23**(6): p. 856-65.
60. Hirsch, F.R., M. Varella-Garcia, and F. Cappuzzo, *Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer.* Oncogene, 2009. **28 Suppl 1**: p. S32-7.
61. Ha, S.Y., et al., *MET overexpression assessed by new interpretation method predicts gene amplification and poor survival in advanced gastric carcinomas.* Mod Pathol, 2013. **26**(12): p. 1632-41.
62. Vogelstein, B., et al., *Cancer genome landscapes.* Science, 2013. **339**(6127): p. 1546-58.
63. Red Brewer, M., et al., *Mechanism for activation of mutated epidermal growth factor receptors in lung cancer.* Proc Natl Acad Sci U S A, 2013. **110**(38): p. E3595-604.
64. Marchetti, A., et al., *EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment.* J Clin Oncol, 2005. **23**(4): p. 857-65.
65. Janne, P.A., J.A. Engelman, and B.E. Johnson, *Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology.* J Clin Oncol, 2005. **23**(14): p. 3227-34.
66. Sharma, S.V., et al., *Epidermal growth factor receptor mutations in lung cancer.* Nat Rev Cancer, 2007. **7**(3): p. 169-81.
67. Takeuchi, K., et al., *RET, ROS1 and ALK fusions in lung cancer.* Nat Med, 2012. **18**(3): p. 378-81.
68. Stransky, N., et al., *The landscape of kinase fusions in cancer.* Nat Commun, 2014. **5**: p. 4846.
69. Du, X., et al., *ALK-rearrangement in non-small-cell lung cancer (NSCLC).* Thorac Cancer, 2018. **9**(4): p. 423-430.

70. Childress, M.A., et al., *ALK Fusion Partners Impact Response to ALK Inhibition: Differential Effects on Sensitivity, Cellular Phenotypes, and Biochemical Properties.* Mol Cancer Res, 2018. **16**(11): p. 1724-1736.
71. Martelli, M.P., et al., *EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues.* Am J Pathol, 2009. **174**(2): p. 661-70.
72. Ross, T.S. and D.G. Gilliland, *Transforming properties of the Huntingtin interacting protein 1/ platelet-derived growth factor beta receptor fusion protein.* J Biol Chem, 1999. **274**(32): p. 22328-36.
73. Wu, Y.M., et al., *Identification of targetable FGFR gene fusions in diverse cancers.* Cancer Discov, 2013. **3**(6): p. 636-47.
74. Ju, Y.S., et al., *A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing.* Genome Res, 2012. **22**(3): p. 436-45.
75. Krasagakis, K., et al., *KIT receptor activation by autocrine and paracrine stem cell factor stimulates growth of merkel cell carcinoma in vitro.* J Cell Physiol, 2011. **226**(4): p. 1099-109.
76. Krystal, G.W., S.J. Hines, and C.P. Organ, *Autocrine growth of small cell lung cancer mediated by coexpression of c-kit and stem cell factor.* Cancer Res, 1996. **56**(2): p. 370-6.
77. Singh, A.B. and R.C. Harris, *Autocrine, paracrine and juxtacrine signaling by EGFR ligands.* Cell Signal, 2005. **17**(10): p. 1183-93.
78. Ferby, I., et al., *Mig6 is a negative regulator of EGF receptor-mediated skin morphogenesis and tumor formation.* Nat Med, 2006. **12**(5): p. 568-73.
79. Gattei, V., et al., *Expression of the RET receptor tyrosine kinase and GDNFR-alpha in normal and leukemic human hematopoietic cells and stromal cells of the bone marrow microenvironment.* Blood, 1997. **89**(8): p. 2925-37.
80. Rao, S.A., et al., *miR-219-5p inhibits receptor tyrosine kinase pathway by targeting EGFR in glioblastoma.* PLoS One, 2013. **8**(5): p. e63164.
81. Paul, M.K. and A.K. Mukhopadhyay, *Tyrosine kinase - Role and significance in Cancer.* Int J Med Sci, 2004. **1**(2): p. 101-115.
82. Pirker, R., et al., *Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial.* Lancet, 2009. **373**(9674): p. 1525-31.
83. Shawver, L.K., D. Slamon, and A. Ullrich, *Smart drugs: tyrosine kinase inhibitors in cancer therapy.* Cancer Cell, 2002. **1**(2): p. 117-23.
84. Forrester, W.C., *The Ror receptor tyrosine kinase family.* Cell Mol Life Sci, 2002. **59**(1): p. 83-96.
85. Stricker, S. and S. Mundlos, *FGF and ROR2 receptor tyrosine kinase signaling in human skeletal development.* Curr Top Dev Biol, 2011. **97**: p. 179-206.
86. Matsuda, T., et al., *Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development.* Mech Dev, 2001. **105**(1-2): p. 153-6.
87. Billiard, J., et al., *The orphan receptor tyrosine kinase Ror2 modulates canonical Wnt signaling in osteoblastic cells.* Mol Endocrinol, 2005. **19**(1): p. 90-101.
88. van Bokhoven, H., et al., *Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome.* Nat Genet, 2000. **25**(4): p. 423-6.
89. Afzal, A.R. and S. Jeffery, *One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinow syndrome and autosomal dominant brachydactyly type B.* Hum Mutat, 2003. **22**(1): p. 1-11.
90. Debebe, Z. and W.K. Rathmell, *Ror2 as a therapeutic target in cancer.* Pharmacol Ther, 2015. **150**: p. 143-8.
91. Cerpa, W., E. Latorre-Esteves, and A. Barria, *RoR2 functions as a noncanonical Wnt receptor that regulates NMDAR-mediated synaptic transmission.* Proc Natl Acad Sci U S A, 2015. **112**(15): p. 4797-802.

92. Davis, E.K., Y. Zou, and A. Ghosh, *Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation.* Neural Dev, 2008. **3**: p. 32.
93. Gomez-Orte, E., et al., *Multiple functions of the noncanonical Wnt pathway.* Trends Genet, 2013. **29**(9): p. 545-53.
94. Roarty, K., et al., *Ror2 regulates branching, differentiation, and actin-cytoskeletal dynamics within the mammary epithelium.* J Cell Biol, 2015. **208**(3): p. 351-66.
95. *Temporal relationships between ovulation and defined changes in the concentration of plasma estradiol-17 beta, luteinizing hormone, follicle-stimulating hormone, and progesterone. I. Probit analysis. World Health Organization, Task Force on Methods for the Determination of the Fertile Period, Special Programme of Research, Development and Research Training in Human Reproduction.* Am J Obstet Gynecol, 1980. **138**(4): p. 383-90.
96. DeChiara, T.M., et al., *Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development.* Nat Genet, 2000. **24**(3): p. 271-4.
97. Yamaguchi, T.P., et al., *A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo.* Development, 1999. **126**(6): p. 1211-23.
98. Bisson, J.A., et al., *Wnt5a and Wnt11 inhibit the canonical Wnt pathway and promote cardiac progenitor development via the Caspase-dependent degradation of AKT.* Dev Biol, 2015. **398**(1): p. 80-96.
99. Okamoto, M., et al., *Noncanonical Wnt5a enhances Wnt/beta-catenin signaling during osteoblastogenesis.* Sci Rep, 2014. **4**: p. 4493.
100. Zhou, Y., T.J. Kipps, and S. Zhang, *Wnt5a Signaling in Normal and Cancer Stem Cells.* Stem Cells Int, 2017. **2017**: p. 5295286.
101. Nemeth, M.J., et al., *Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation.* Proc Natl Acad Sci U S A, 2007. **104**(39): p. 15436-41.
102. Maeda, K., et al., *Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis.* Nat Med, 2012. **18**(3): p. 405-12.
103. Akbarzadeh, S., et al., *The deleted in brachydactyly B domain of ROR2 is required for receptor activation by recruitment of Src.* PLoS One, 2008. **3**(3): p. e1873.
104. Nishita, M., et al., *Cell/tissue-tropic functions of Wnt5a signaling in normal and cancer cells.* Trends Cell Biol, 2010. **20**(6): p. 346-54.
105. Cheung, R., J. Kelly, and R.J. Macleod, *Regulation of villin by wnt5a/ror2 signaling in human intestinal cells.* Front Physiol, 2011. **2**: p. 58.
106. Yu, J., et al., *Wnt5a induces ROR1/ROR2 heterooligomerization to enhance leukemia chemotaxis and proliferation.* J Clin Invest, 2016. **126**(2): p. 585-98.
107. Hikasa, H., et al., *The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling.* Development, 2002. **129**(22): p. 5227-39.
108. Ford, C.E., et al., *The dual role of the novel Wnt receptor tyrosine kinase, ROR2, in human carcinogenesis.* Int J Cancer, 2013. **133**(4): p. 779-87.
109. O'Connell, M.P., et al., *The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma.* Oncogene, 2010. **29**(1): p. 34-44.
110. Morioka, K., et al., *Orphan receptor tyrosine kinase ROR2 as a potential therapeutic target for osteosarcoma.* Cancer Sci, 2009. **100**(7): p. 1227-33.
111. Ren, D., et al., *Wnt5a induces and maintains prostate cancer cells dormancy in bone.* J Exp Med, 2019. **216**(2): p. 428-449.
112. Wright, T.M., et al., *Ror2, a developmentally regulated kinase, promotes tumor growth potential in renal cell carcinoma.* Oncogene, 2009. **28**(27): p. 2513-23.

113. Lara, E., et al., *Epigenetic repression of ROR2 has a Wnt-mediated, pro-tumourigenic role in colon cancer.* Mol Cancer, 2010. **9**: p. 170.
114. Sun, C., R. Mezzadra, and T.N. Schumacher, *Regulation and Function of the PD-L1 Checkpoint.* Immunity, 2018. **48**(3): p. 434-452.
115. Ishida, Y., et al., *Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death.* EMBO J, 1992. **11**(11): p. 3887-95.
116. Escors, D., et al., *The intracellular signalosome of PD-L1 in cancer cells.* Signal Transduct Target Ther, 2018. **3**: p. 26.
117. Wu, Y., et al., *PD-L1 Distribution and Perspective for Cancer Immunotherapy-Blockade, Knockdown, or Inhibition.* Front Immunol, 2019. **10**: p. 2022.
118. Zhang, J., et al., *Biochemical Aspects of PD-L1 Regulation in Cancer Immunotherapy: (Trends in Biochemical Sciences 43, 1014-1032; 2018).* Trends Biochem Sci, 2019. **44**(6): p. 557.
119. Kim, D.H., et al., *Exosomal PD-L1 promotes tumor growth through immune escape in non-small cell lung cancer.* Exp Mol Med, 2019. **51**(8): p. 94.
120. Gong, B., et al., *Secreted PD-L1 variants mediate resistance to PD-L1 blockade therapy in non-small cell lung cancer.* J Exp Med, 2019. **216**(4): p. 982-1000.
121. Wang, Q., F. Liu, and L. Liu, *Prognostic significance of PD-L1 in solid tumor: An updated meta-analysis.* Medicine (Baltimore), 2017. **96**(18): p. e6369.
122. Salmaninejad, A., et al., *PD-1/PD-L1 pathway: Basic biology and role in cancer immunotherapy.* J Cell Physiol, 2019. **234**(10): p. 16824-16837.
123. Brahmer, J.R., et al., *Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates.* J Clin Oncol, 2010. **28**(19): p. 3167-75.
124. Gong, J., et al., *Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations.* J Immunother Cancer, 2018. **6**(1): p. 8.
125. Teo, P.Y., et al., *Ovarian cancer immunotherapy using PD-L1 siRNA targeted delivery from folic acid-functionalized polyethylenimine: strategies to enhance T cell killing.* Adv Healthc Mater, 2015. **4**(8): p. 1180-9.
126. Luo, X., et al., *Folic acid-functionalized polyethylenimine superparamagnetic iron oxide nanoparticles as theranostic agents for magnetic resonance imaging and PD-L1 siRNA delivery for gastric cancer.* Int J Nanomedicine, 2017. **12**: p. 5331-5343.
127. Kuruma, H., et al., *A novel antiandrogen, Compound 30, suppresses castration-resistant and MDV3100-resistant prostate cancer growth in vitro and in vivo.* Mol Cancer Ther, 2013. **12**(5): p. 567-76.
128. Bishop, J.L., et al., *The Master Neural Transcription Factor BRN2 Is an Androgen Receptor-Suppressed Driver of Neuroendocrine Differentiation in Prostate Cancer.* Cancer Discov, 2017. **7**(1): p. 54-71.
129. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.* Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.
130. Wyatt, A.W., et al., *Heterogeneity in the inter-tumor transcriptome of high risk prostate cancer.* Genome Biol, 2014. **15**(8): p. 426.
131. Gao, J., et al., *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal, 2013. **6**(269): p. pl1.
132. Lin, D., et al., *Next generation patient-derived prostate cancer xenograft models.* Asian J Androl, 2014. **16**(3): p. 407-12.

133. Zaidi, M.R. and G. Merlino, *The two faces of interferon-gamma in cancer.* Clin Cancer Res, 2011. **17**(19): p. 6118-24.
134. Ayers, M., et al., *IFN-gamma-related mRNA profile predicts clinical response to PD-1 blockade.* J Clin Invest, 2017. **127**(8): p. 2930-2940.
135. Danaher, P., et al., *Pan-cancer adaptive immune resistance as defined by the Tumor Inflammation Signature (TIS): results from The Cancer Genome Atlas (TCGA).* J Immunother Cancer, 2018. **6**(1): p. 63.
136. Bishop, J.L., et al., *PD-L1 is highly expressed in Enzalutamide resistant prostate cancer.* Oncotarget, 2015. **6**(1): p. 234-42.
137. Fertig, E.J., et al., *Gene expression signatures modulated by epidermal growth factor receptor activation and their relationship to cetuximab resistance in head and neck squamous cell carcinoma.* BMC Genomics, 2012. **13**: p. 160.
138. Peraldo-Neia, C., et al., *Epidermal Growth Factor Receptor (EGFR) mutation analysis, gene expression profiling and EGFR protein expression in primary prostate cancer.* BMC Cancer, 2011. **11**: p. 31.
139. Goodspeed, A., et al., *A Gene Expression Signature Predicts Bladder Cancer Cell Line Sensitivity to EGFR Inhibition.* Bladder Cancer, 2018. **4**(3): p. 269-282.
140. Asem, M.S., et al., *Wnt5a Signaling in Cancer.* Cancers (Basel), 2016. **8**(9).
141. Tsujimura, T., et al., *Ligand-independent activation of c-kit receptor tyrosine kinase in a murine mastocytoma cell line P-815 generated by a point mutation.* Blood, 1994. **83**(9): p. 2619-26.
142. Guo, G., et al., *Ligand-Independent EGFR Signaling.* Cancer Res, 2015. **75**(17): p. 3436-41.
143. Zerdes, I., et al., *Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations.* Oncogene, 2018. **37**(34): p. 4639-4661.