

Deciphering the independent roles of *PRUNE2* and *PCA3* in the function of the *PRUNE2/PCA3/AR* triad and prostate cancer progression

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DECIPHERING THE INDEPENDENT ROLES OF *PRUNE2* AND *PCA3* IN THE FUNCTION OF THE *PRUNE2/PCA3/AR* TRIAD AND PROSTATE CANCER PROGRESSION

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Quando vier a primavera,
Se eu já estiver morto,
As flores florirão da mesma maneira
E as árvores não serão menos verdes que na primavera passada.
A realidade não precisa de mim.

Sinto uma alegria enorme
Ao pensar que a minha morte não tem importância nenhuma.

Se soubesse que amanhã morria
E a primavera era depois de amanhã,
Morreria contente, porque ela era depois de amanhã.
Se esse é o seu tempo, quando havia ela de vir senão no seu tempo?
Gosto que tudo seja real e que tudo esteja certo;
E gosto porque assim seria, mesmo que eu não gostasse.
Por isso, se morrer agora, morro contente,
Porque tudo é real e tudo está certo.

Podem rezar latim sobre o meu caixão, se quiserem.
Se quiserem, podem dançar e cantar à roda dele.
Não tenho preferências para quando já não puder ter preferências.
O que for, quando for, é que será o que é.

- Alberto Caeiro “Quando vier a primavera”

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RELEVANT ABBREVIATIONS

aa – Amino Acids

ADT – Androgen Deprivation Therapy

AKT – Protein Kinase B

AR – Androgen Receptor

ATM – Ataxia-Telangiectasia Mutated

Bcl-2 – B-cell lymphoma 2

BCH – BNIP2 and Cdc42GAP Homology

BIM – Bcl-2 Interacting Mediator of cell death

BNIP2 – Bcl-2 / adenovirus E1B 19 kDa-interacting protein 2

BPH – Benign Prostatic Hyperplasia

BRCA1 – Breast Cancer gene 1

BRCA2 – Breast Cancer gene 2

BRIP1 – *BRCA1*-interacting protein C-terminal helicase 1

CaP – Cancro da Próstata

Cdc42GA – Cell division control protein 42 homolog GTPase-activating protein

cDNA – Complementary DNA

CHEK2 – Checkpoint Kinase 2

Ct – Cycle threshold

DEG – Differentially Expressed Genes

DRE – Digital Rectal Examination

DDR – DNA Damage Response

DHT – 5 α -dihydrotestosterone

EAU – European Association of Urology

ECM – Extracellular Matrix

EMT – Epithelial-Mesenchymal Transition

ESMO – European Society for Medical Oncology

FDA – US Food and Drug Administration

FDR – False Discovery Rate

FOXO3a – Forkhead Box O3

GAPs – GTPase-activating proteins

GEFs – Guanine nucleotide exchange factors

GO – Gene Ontology

GUSB – Beta glucuronidase

HPC – Hereditary Prostate Cancer

HOXB13 – Homeobox B13

IF – Immunofluorescence

IGFBP7 – Insulin-like growth factor binding protein-7

KEGG – Kyoto Encyclopedia of Genes

kDa – Kilodalton

LAMC2 – Laminin subunit γ 2

lncRNAs – Long Noncoding RNAs

LS – Lynch Syndrome

mHSPC – metastatic hormone-sensitive PrCa

mCRPC – metastatic castration-resistant PrCa

MMR – Mismatch Repair

MLH1 – MutL Homolog 1

MSH2 – MutS Homolog 2

MSH6 – MutS Homolog 6

MYC – Myelocytomatosis oncogene

NBS1 – Nijmegen Breakage Syndrome 1

NCCN – National Comprehensive Cancer Network

NGS – Next generation sequencing

NKX3.1 – NK3 homeobox 1

PALB2 – Partner and Localizer of BRCA2

PARP – Poly (ADP-ribose) Polymerase

PBS – Phosphate-Buffered Saline

PCA3 – Prostate Cancer Antigen 3

PI3K – Phosphoinositide 3-kinase

PI – Propidium Iodide

PIN – Prostatic Intraepithelial Neoplasia

PMS2 – PMS1 Homolog 2

PrCa – Prostate Cancer

PRUNE2 – Prune Homolog 2 with BCH domain

PSA – Prostate-Specific Antigen

PTEN – Phosphatase and Tensin Homolog

qRT-PCR – quantitative Real-Time Polymerase Chain Reaction

RB1 – Retinoblastoma 1

ROI – Region of Interest

RR – Relative Risk

SDS-PAGE – SDS-polyacrylamide gel

siRNA – Short interfering RNA

THBS4 – Thrombospondin 4

TME – Tumor Microenvironment

TMPRSS2 – Transmembrane protease serine 2

TNM – Tumor, node and metastasis stage

TP53 – Tumor Protein 53

WB – Western Blot

WES – Whole-Exome Sequencing

WP – WikiPathways

ABSTRACT

Prostate Cancer (PrCa) is one of the leading causes of cancer incidence and mortality in men worldwide. Family history is one of the most relevant risk factors associated with this disease, however, the mechanisms responsible for the increase in PrCa susceptibility are still elusive. Previously, our group performed whole-exome sequencing in 96 affected family members belonging to 45 families and reported, for the first time, pathogenic/likely pathogenic germline variants in the tumor suppressor gene *PRUNE2* in hereditary and/or early onset PrCa patients, leading to its proposal as a novel biomarker for PrCa predisposition. *PRUNE2*'s deregulation has previously been reported in several other cancer types such as neuroblastoma, colorectal adenocarcinoma, and leiomyosarcoma, but its role in PrCa may be more complex. Particularly, in PrCa, *PRUNE2* is negatively regulated by a long-noncoding RNA, *PCA3*, which acts as an oncogene. Based on these previous findings, the main objective of this project was to further investigate the regulatory axis between *PRUNE2* and *PCA3* in PrCa, as well as *PRUNE2*'s involvement with other deregulated genes and signaling pathways.

Our comprehensive transcriptomic analysis revealed 86 differentially expressed genes in *PRUNE2* variant carriers (40 upregulated and 46 downregulated), most of them associated with signaling pathways related to the extracellular matrix. Furthermore, some of the identified genes (*LAMC2*, *IGFBP7*, *THBS4*) are also associated with the *PI3K/Akt* pathway, often deregulated in cancer. Additionally, siRNA-mediated knockdown of *PRUNE2* and *PCA3*, established in androgen-sensitive prostate cell lines (LNCaP and PNT1A), showed a distinct isoform profile of *PRUNE2* between the tumorigenic and non-tumorigenic lines, respectively. Immunofluorescence assays on these cell lines revealed a different *PRUNE2* subcellular localization between LNCaP and PNT1A. In the tumorigenic cell line, *PRUNE2* is mostly localized in the cytoplasm, while in the non-tumorigenic cell line, it is predominantly localized in the nucleoli. These findings indicate that *PRUNE2* may have a nuclear function in non-tumorigenic cells, that is altered upon malignant transformation. Importantly, these results suggest that *PRUNE2*'s localization and function are regulated via different mechanisms in normal versus cancer cells.

With this study, we have advanced our understanding of *PRUNE2*'s role in PrCa, highlighting this gene's involvement in paramount oncogenic signaling pathways, and potentially opening new avenues for specific management strategies and targeted therapies.

RESUMO

O cancro da próstata (CaP) é uma das principais causas de incidência e mortalidade por cancro nos homens em todo o mundo. A história familiar é um dos fatores de risco mais relevantes associados a esta doença, no entanto, os mecanismos responsáveis pelo aumento da suscetibilidade para o desenvolvimento de CaP são ainda pouco conhecidos. Anteriormente, o nosso grupo de investigação realizou a sequenciação completa do exoma em 96 indivíduos afetados, pertencentes a 45 famílias e reportou, pela primeira vez, variantes germinativas patogénicas/possivelmente patogénicas no gene supressor tumoral *PRUNE2*, em doentes com CaP hereditário e/ou de idade jovem. Assim, este gene foi proposto como um novo biomarcador de predisposição para CaP. A desregulação do *PRUNE2* foi previamente descrita em vários outros tipos de cancro, como o neuroblastoma, o cancro colorretal e o leiomiossarcoma, mas o seu papel torna-se mais complexo em CaP. Em particular, em CaP, o *PRUNE2* é negativamente regulado por um RNA não-codificante, o *PCA3*, que atua como um oncogene. Com base nas observações anteriores, o objetivo principal deste projeto consistiu em investigar a correlação entre o *PRUNE2* e o *PCA3* em CaP, em particular, o seu impacto na desregulação de outros genes e vias de sinalização oncogénicas.

A nossa análise transcriptómica revelou 86 genes diferencialmente expressos em portadores de variantes no *PRUNE2* (40 regulados positivamente e 46 regulados negativamente), a maioria dos quais associados a vias de sinalização relacionadas com a matriz extracelular. Além disso, alguns dos genes (*LAMC2*, *IGFBP7*, *THBS4*) encontram-se também associados à via *PI3K/Akt*, frequentemente desregulada em cancro. Adicionalmente, o *knockdown* mediado por siRNA do *PRUNE2* e do *PCA3*, estabelecido em linhas celulares de próstata sensíveis a androgénios, mostrou um perfil distinto de isoformas de *PRUNE2* entre as linhas tumorigénicas (LNCaP) e não tumorigénicas (PNT1A). Os ensaios de imunofluorescência nestas linhas celulares revelaram uma localização subcelular diferente do *PRUNE2* entre LNCaP e PNT1A. Na linha tumorigénica, o *PRUNE2* encontra-se localizado maioritariamente no citoplasma, enquanto na linha não tumorigénica, é expresso predominantemente nos nucléolos. Estes resultados indicam que o *PRUNE2* pode ter uma função nuclear específica nas células não tumorigénicas, que é alterada durante a transformação maligna, sugerindo que a sua localização e função poderão ser reguladas por mecanismos diferentes entre células normais e células cancerígenas.

Com este estudo, contribuímos para uma melhor compreensão da função do *PRUNE2* em CaP, revelando o envolvimento deste gene em vias de sinalização oncogénicas importantes, abrindo assim novas perspetivas para o desenvolvimento de estratégias de gestão clínica personalizadas e terapias direcionadas.

INTRODUCTION

1. Prostate

1.1. Anatomy and Histology

The prostate gland is an organ located adjacent to the urethra, below the bladder, and normally weighs between 15 and 20 g (Ittmann, 2018; Rebello et al., 2021). In adult men, it is divided into central, transition, and peripheral zones (Figure 1A) (Sandhu et al., 2021).

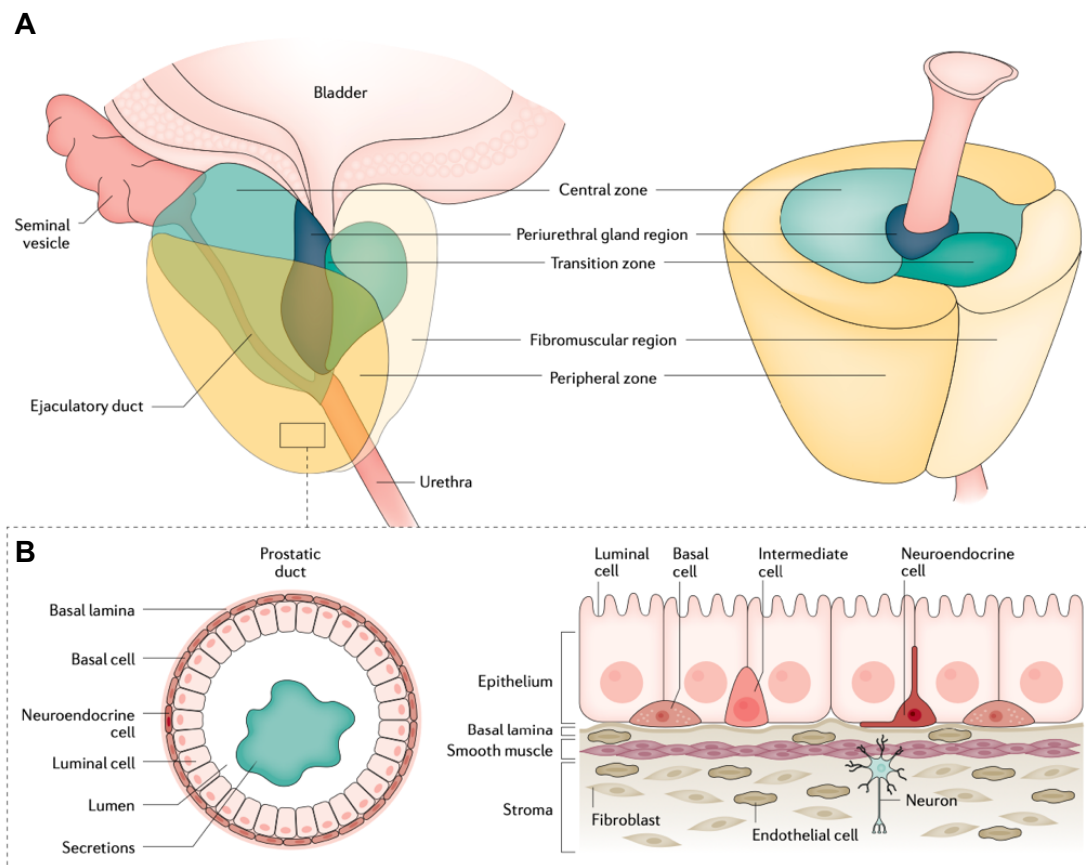


Figure 1: Anatomical location and cellular composition of the prostate gland [A] The prostate is located below the bladder and is divided into central, transition and peripheral zones. [B] Histological structure of the prostate, illustrating the different cell types (basal, neuronal and luminal) that compose the prostate gland. Adapted from (Rebello et al., 2021).

The peripheral zone, which envelops the prostatic urethra and composes the outer part of the gland, makes up for over 70% of all prostatic tissue. It is also the area where the majority of prostate tumors originate in older men (Ittmann, 2018; Rebello et al., 2021). The transition zone is the area closest to the prostatic urethra, accounting for only around 5% of the entire area of the prostate in younger men (Ittmann, 2018). However, as men age, the transition zone increases in volume due to increased cell proliferation. Importantly, it is in

the transition zone that benign prostatic hyperplasia (BPH), a common lesion in older men, may arise (Cannarella et al., 2021). Besides BPH, cancer can also arise in the transition zone, accounting for 20% of all cases (Lee et al., 2015). However, there are differences between these and the ones developed in the peripheral zone (Ittmann, 2018). Specifically, tumors in the transition zone are normally larger when diagnosed and harder to detect due to their central location (Sakai et al., 2006). Nonetheless, tumors arising from the transition zone are less aggressive than those in the peripheral zone and, due to their lower cell proliferation rates and lower Gleason scores, are associated with a better clinical outcome (Lee et al., 2015). Lastly, the central zone, located between the transition and peripheral zones, surrounds the ejaculatory ducts (Ittmann, 2018). Malignant lesions from this zone, although not regularly reported, equate to more aggressive tumors than the ones outside of the central zone (Vargas, 2012). In addition to the three different zones, the prostate also contains periurethral and fibromuscular regions. The periurethral gland region is involved around the prostatic urethra, while the fibromuscular region, also known as the stroma, plays a crucial role in supporting the epithelial layer (Rebello et al., 2021). During cancer development, the stroma becomes reactive and displays an increase in vimentin and fibroblast expression, therefore, playing an important role in altering the tumor microenvironment (TME) and aiding in cancer development (Pederzoli et al., 2023).

Microscopically, the normal prostate gland can be divided into two main sections, the epithelium and the stroma (Verze et al., 2016) (Figure 1B). The epithelium is composed of ducts and acini, which are surrounded by the stroma (Ittmann, 2018). The acini consist of cells that secrete prostatic fluid into the ducts, which then transport it into the urethra (Rebello et al., 2021; Verze et al., 2016). Three different types of cells constitute the acini and the ducts: luminal, basal, and neuroendocrine cells (Wang et al., 2018). The luminal cells, columnar in shape with the nuclei located near the bottom of the cell (Ittmann, 2018), are mainly involved in producing several products such as the prostate-specific antigen (PSA), and secreting them into the prostatic fluid (Verze et al., 2016). This layer is surrounded by basal, intermediate, and neuroendocrine cells, which form the basement membrane (Rebello et al., 2021). The basal cells form the outer layer of cells in contact with the stroma and aid in structural support and proliferation. Neuroendocrine cells, which only represent about 1% of the entire epithelial cell population, are responsible for the maintenance of the TME and response to stimuli (Butler & Huang, 2021). The other section of the prostate gland, the stroma, consists of smooth muscle cells and fibroblasts, which support the prostatic ducts (Rebello et al., 2021; Verze et al., 2016).

1.2. Prostatic lesions

Prostatic lesions are commonly diagnosed in men with more advanced age. Among them, the most common is BPH, in which there is an increase in the proliferation of epithelial cells from the transition zone, leading to prostate enlargement (Cannarella et al., 2021). The development and progression of BPH are linked to aging, hormonal changes, and prostatic inflammation (Verze et al., 2016). Hormonal changes, namely decreased testosterone production, can also lead to the inflammation of the prostate (Cannarella et al., 2021). In this condition, there is an increase in infiltration of T and B lymphocytes and macrophages in the prostate, which stimulate cell proliferation, causing damage to epithelial and stromal cells (Verze et al., 2016). Men affected by BPH can experience an increased need to urinate, difficulty in starting urination, and a sensation of incomplete bladder emptying, among other symptoms, due to the obstruction of the prostatic urethra (Langan, 2019).

Although BPH is a benign condition, cellular damage and an increase in inflammation can contribute to the development of malignant prostatic lesions. These, like prostatic intraepithelial neoplasia (PIN), arise from basal or luminal cells that have undergone genetic mutations (Rebello et al., 2021). These cells proliferate excessively as a result of chronic inflammation and infections, as well as prostate enlargement, and can induce a state of hypoxia which leads to the generation of reactive oxygen species that induce DNA mutations (Sandhu et al., 2021; Verze et al., 2016). Furthermore, the oxidative stress can induce a shortening of the telomeres on chromosomes, which may lead to chromosomal instability (Shen & Abate-Shen, 2010). These events result in the formation of PIN lesions, in which there is an increase in luminal cells, along with a decrease in basal cells (Figure 2) (Sandhu et al., 2021). PIN lesions are considered precursors of prostatic adenocarcinomas and are classified as premalignant (Rebello et al., 2021). These lesions can be categorized as low-grade or high-grade. The process typically begins with the transformation of normal epithelium into low-grade PIN, which then progresses to high-grade PIN, and ultimately develops into a carcinoma (De Marzo et al., 2016). In addition to the shortening of the telomeres, high-grade PIN lesions share several genetic markers with adenocarcinomas. These markers include the loss of one allele in chromosome 8p12-21 (Brawer, 2005), the loss of basal markers such as p63 and cytokeratins 5 and 14, as well as the gain of luminal markers like cytokeratins 8 and 18 (Sandhu et al., 2021). Once established, localized prostate cancer (PrCa) may progress to become locally invasive and lastly into metastatic PrCa (Wang et al., 2018).

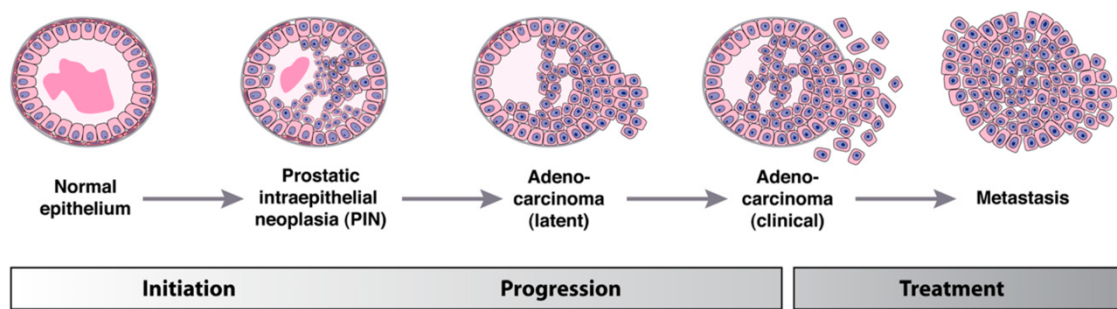


Figure 2: Progression of the normal prostatic epithelium until the development of metastasis. The normal prostate gland acquires genetic mutations due to inflammation and infections, which result in the development of PIN. These lesions develop into adenocarcinoma and, eventually, into the formation of metastasis. Adapted from (Shen & Abate-Shen, 2010).

Adenocarcinomas can be divided into two different types, acinar and non-acinar. Acinar adenocarcinomas, which account for about 95% of all PrCa cases, originate from epithelial cells and develop in the acini (Alizadeh & Alizadeh, 2014). These tumors can be detected during a digital rectal exam (DRE) and are associated with an increase in PSA levels (Baig et al., 2015). Among the non-acinar, the most common subtypes are the intraductal adenocarcinoma and the ductal adenocarcinoma (Rebello et al., 2021). The first is characterized by the abnormal proliferation of epithelial cells in the ducts and acini, with the basal cells remaining intact (Humphrey, 2017). On the other hand, the latter, also known as papillary adenocarcinoma, originates from the cells lining the ducts and generally has a worse prognosis compared to acinar adenocarcinomas (Humphrey, 2012; Rebello et al., 2021).

2. Prostate Cancer

2.1. Epidemiology

PrCa ranks second among the most incident cancers worldwide, with over 1.4 million new cases in 2020, representing 15.1% of all cancer incidence in men (World Cancer Research Fund International, 2018). As for global cancer-related deaths, PrCa is also one of the leading causes, with more than 350.000 deaths annually (Rebello et al., 2021). In Portugal, as well as in the European Union, this cancer type takes the first spot as the most common and the third deadliest overall (Figure 3) (Joint Research Centre, 2022).

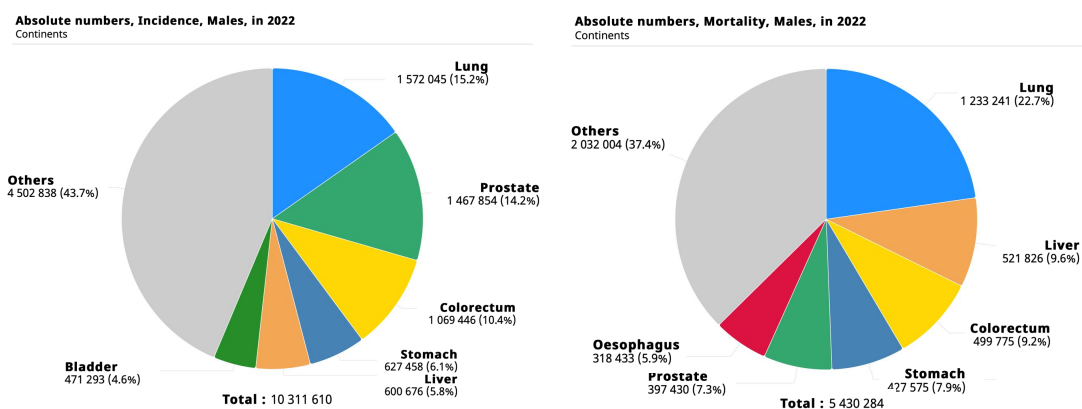


Figure 3: Absolute incidence and mortality numbers depicting the most common cancers in males, in 2022. Adapted from (Globocan, 2020).

The incidence of PrCa is higher in more developed regions such as Europe, North America, Australia, and New Zealand. In contrast, the incidence is lower in generally less developed countries located in Central and South Asia, as well as in Africa, although there has been a recent rise in newly diagnosed cases (Rebello et al., 2021).

2.2. Etiology

The most well-established risk factors associated with PrCa are limited to age, family history and genetic predisposition, as well as ethnicity (Ng, 2021). Although factors such as alcohol and tobacco consumption, obesity, or an unbalanced diet have not been conclusively associated with an increased risk of PrCa (Ng, 2021), there is evidence suggesting that these factors may also play a role in PrCa mortality (Rebello et al., 2021). Regarding age, there is a strong correlation between advancing age and the increased risk of developing this disease, with over 85% of diagnosed cases globally belonging to individuals older than 60 years [1]. In fact, the average age of diagnosis stands at 66 years

old [22]. This age is lower when looking at individuals with a positive family history of PrCa, particularly in men with a first-degree relative diagnosed with this disease before the age of 66 years old (Figure 4) [23].

Interestingly, a racial bias in the development of PrCa has been reported, with men of African ancestry consistently being associated with a higher relative risk (RR) for developing an early and more aggressive form of the disease (Zeigler-Johnson, 2008). These differences may be due behavioral, environmental, or even cultural factors, but are primarily linked to genetic variations between populations (Brandao et al., 2020; Cardoso et al., 2023; Rebbeck, 2018).

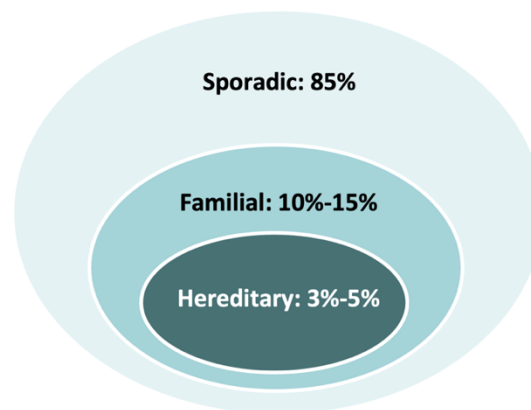


Figure 4: Representation of the percentage of prostate cancer cases that are considered sporadic, familial and hereditary. Adapted from (Ni Raghallaigh & Eeles, 2022).

Additionally, around 10-15% of men diagnosed with PrCa have a family history of the disease (Grozescu T, 2017). Men with close relatives who have been diagnosed with PrCa have a two to four-fold increased risk of developing this disease, compared to those with no family history (Ng, 2021; Sekhoacha et al., 2022; Vietri et al., 2021). This risk further increased with the number of affected family members (Brandao et al., 2020). Moreover, if the affected relative is a first-degree family member, such as a brother or a father, the RR for developing PrCa increases to 3.1- and 2.4-fold, respectively (Zhen et al., 2018).

2.3. Diagnosis

One of the major challenges in diagnosing PrCa lies in the difficulty of distinguishing between indolent tumors, unlikely to pose a threat, and aggressive ones with the potential to metastasize (Shafi et al., 2013).

According to the National Comprehensive Cancer Network (NCCN) guidelines for PrCa early detection, not all patients diagnosed with this disease need to undergo treatment and can instead remain under surveillance (Figure 5) (National Comprehensive Cancer Network, 2024b). Thus, when evaluating a patient, several considerations need to be taken into account to perform a risk assessment. These include the patient's family cancer history, such as first- or second-degree relatives with a history of metastatic PrCa, male breast cancer, female breast cancer in individuals aged 45 years or younger, colorectal or endometrial cancers in individuals aged 50 years or younger, pancreatic cancer, or two or more relatives with colorectal, endometrial, breast or PrCa diagnosed at any age. Furthermore, factors to consider include the patient's ethnicity or medications taken that decrease the *PSA* levels (National Comprehensive Cancer Network, 2024b).

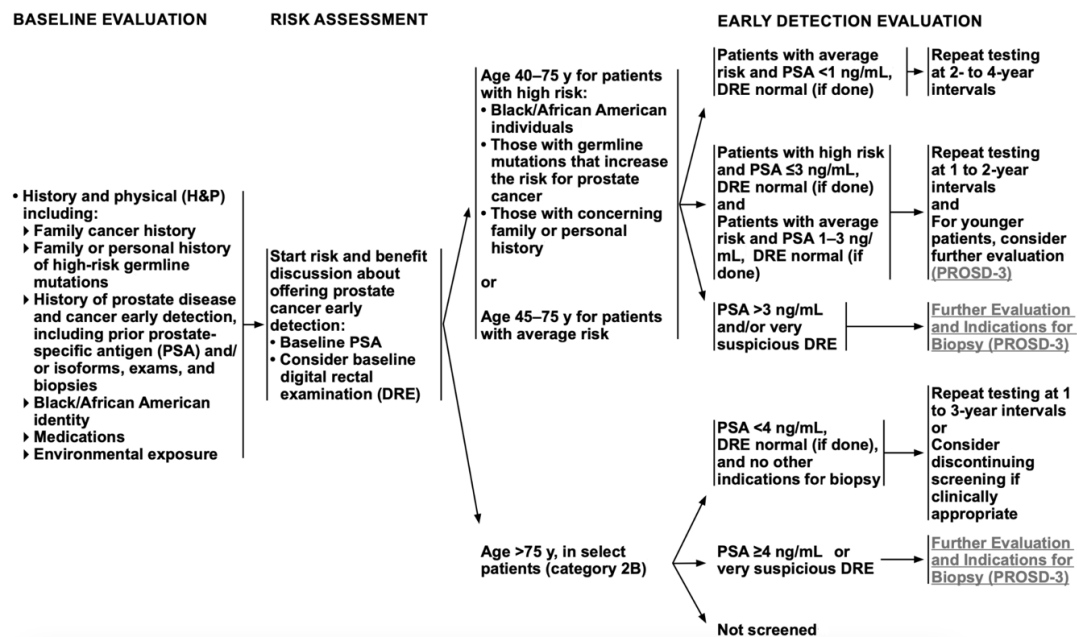


Figure 5: NCCN guidelines for the early detection of PrCa, based on a baseline evaluation, risk assessment and early detection evaluation. Adapted from (National Comprehensive Cancer Network, 2024a).

2.3.1. PSA

The first line of detection composes of evaluating the serum *PSA* present in the blood, in patients between the ages of 40 and 75. *PSA* is a glycoprotein produced by the epithelial cells of the prostate and is detected in the prostatic fluid (Sekhoacha et al., 2022). In healthy cells, *PSA* is secreted into the prostatic ducts. However, in PrCa, there is a leakage of *PSA* into the extracellular space, causing an increase of this protease (Akizhanova et al., 2017; Vietri et al., 2021). In addition to the seminal fluid, *PSA* is also

released into the bloodstream, allowing for the detection of abnormal levels caused by inflammation or neoplasia (Akizhanova et al., 2017; Shafi et al., 2013).

The most standard cutoff for *PSA* in blood samples of healthy individuals is 4.0 ng/mL (Sekhoacha et al., 2022). In cases where *PSA* levels increase > 4.0 ng/mL, a biopsy is generally recommended to confirm the presence of PrCa (Vietri et al., 2021). Routine *PSA* screening is advised in patients between the ages of 40 and 75. In case of *PSA* ≤ 3 ng/mL for patients belonging to an average or high-risk group, vigilance and repeated testing 2-4 years or 1-2 years, respectively, is recommended. If the score is > 3 ng/mL and/or the DRE exam raises suspicion, a biopsy is typically requested (National Comprehensive Cancer Network, 2024b). For older patients that appear healthy and don't have any comorbidities, *PSA* testing can also be performed, with only values of serum *PSA* ≥ 4 ng/mL being considered for biopsy (National Comprehensive Cancer Network, 2024b). The lack of specificity in diagnosing PrCa often leads to false positives, overdiagnosis, and potential overtreatment. This highlights the necessity for identifying and characterizing new biomarkers in PrCa to improve risk stratification and early detection (Sandhu et al., 2021; Sekhoacha et al., 2022).

The recommended age for *PSA* screening varies between the United States and Europe. According to the American Urological Association, *PSA* screening should start at age 55 and continue until age 68. Meanwhile, the European Association of Urology (EAU) proposes starting screening between ages 40 and 45 (Heidenreich et al., 2013). Screening for PrCa using *PSA* levels can increase life expectancy in men with localized PrCa (Rebello et al., 2021). According to the American Cancer Society, if *PSA* levels rise between 4.0 ng/mL and 10 ng/mL, the chances of having PrCa are around 25%, and they increase to over 50% if the *PSA* value is over 10 ng/mL (Akizhanova et al., 2017; Sekhoacha et al., 2022). There's an ongoing debate about the accuracy of this screening. While it has contributed to reduced mortality rates by enabling early PrCa detection, it has a poor specificity, ranging from 20-40%, leading to high rates of false positives (Adamaki & Zoumpourlis, 2021). *PSA* levels can be affected by several factors such as older age, higher body mass index, or inflammation of the prostate gland (Akizhanova et al., 2017). Furthermore, while *PSA* is prostate gland specific, it is not PrCa-specific, therefore, increased levels can also indicate benign pathologies, such as BPH (Sandhu et al., 2021; Sekhoacha et al., 2022).

2.3.2. Digital rectal examination

The DRE has been used in combination with *PSA* as the first step in screening for PrCa in a primary health care setting (Naji et al., 2018). DRE involves inserting a gloved finger into the rectum to evaluate the condition of the prostate gland (Sekhoacha et al., 2022). If any abnormalities are felt during the examination, a biopsy may be recommended (Adamaki & Zoumpourlis, 2021). Similar to the *PSA* test, there is controversial evidence regarding the effectiveness of DRE. This test has a high specificity (83.6%), effectively identifying men without PrCa, but its sensitivity is low (53.2%), which can lead to under-diagnosis of some cancers (Naji et al., 2018).

2.3.3. Biopsy

A biopsy is imperative in detecting malignancy in the prostate. The biopsy is recommended if *PSA* levels are abnormally high, or if the DRE examination raises suspicion (Rebello et al., 2021). During a biopsy, a small needle is used to collect samples from several areas of the prostate. This procedure is guided by transrectal ultrasound, which uses sound waves to locate and visualize the prostate gland (Sekhoacha et al., 2022). The collected samples are then analyzed under a microscope by a pathologist to check for the presence of malignant cells. The diagnosis considers the clinical stage, Gleason score, and *PSA* levels, and categorizes the tumor as low, intermediate, or high risk (Litwin & Tan, 2017; Mohler et al., 2016).

2.3.4. Histopathological grading and Gleason Score

The Gleason Score system, is used to grade PrCa. This grading system was developed to communicate the risk associated with the disease, and to guide patients in making informed treatment decisions (Tagai et al., 2019). The Gleason Score was first introduced in the 1960s to classify prostate tumors based on their glandular architecture (Adamaki & Zoumpourlis, 2021). It consists of five grades, with grade one representing the highest level of differentiation and grade five representing the lowest level of differentiation (Humphrey, 2004). Given PrCa heterogeneity, the two most predominant histological patterns are reported and summed, allowing the classification of these tumors into low (≤ 6), intermediate (7) or high (8 to 10) Gleason Score (Lotan & Epstein, 2010). The Gleason Score currently used has been slightly modified, with tissue samples now being graded only from 3 to 5, as grades 1 and 2 are rarely used and are typically indicative of BPH and not

malignancy (Adamaki & Zoumpourlis, 2021; Epstein et al., 2016). This method presents some challenges, as it may cause unnecessary fear in patients. Since a score of 6 is the lowest possible, it can be perceived as an intermediate grade, leading to believe that a treatment is necessary (Epstein et al., 2016). Furthermore, the usual classification that divides the scores into low, intermediate or high compiles two different types of classification into a Gleason Score of 7 (Epstein et al., 2014). Cancers with a score 3 + 4 have a better prognosis than the ones with a score of 4 + 3. This is because the former indicates a predominantly well-differentiated cancer, while the latter is used for a mostly poorly differentiated cancer (Epstein et al., 2016).

The most recent version of this grading system divides tumors into 5 graded groups, accordingly: Group 1 represents Gleason Scores < 6; Group 2 for Gleason Scores 3 + 4 = 7; Group 3 with Gleason Scores 4 + 3 = 7; Group 4 for Gleason Score = 8; lastly, Group 5 with Gleason Scores = 9 and 10 (Epstein et al., 2016). This new grading system is currently being combined with the original Gleason Score and has been accepted by the World Health Organization (Epstein et al., 2014).

2.3.5. PrCa staging and risk assessment

PrCa is separated into different staging groups to aid professionals in communicating the best treatment options available to the patient, as well as evaluate the severity of the disease and predicting a prognosis (Buyyounouski et al., 2017).

The tumor, node and metastasis (TNM) staging is used internationally to evaluate the extent of the primary tumor, the presence of affected lymph nodes and the presence of distant metastasis (Amin et al., 2017). To further distinguish the disease, the TNM system separates PrCa according to the clinical and pathological staging (Table 1). Clinical staging uses diagnostic tools like the DRE and magnetic resonance imaging (MRI) to aid in guiding treatment decisions. Meanwhile, pathological staging incorporates the patients information obtained from the clinic, as well as surgical finds from a prostatectomy (Amin et al., 2017). Since not all patients go through a prostatectomy, sometimes the pathological staging is not available (Varma et al., 2019). Furthermore, the TNM staging is used alongside an evaluation of *PSA* levels and tumor grading to determine the prognostic stage groups (Table 2) (Buyyounouski et al., 2017).

Table 1: PrCa staging from the American Joint Committee on Cancer TNM Criteria. Adapted from (Buyyounouski et al., 2017).

Clinical (cT)
T category
TX – Primary tumor cannot be assessed
T0 – No evidence of primary tumor
T1 – Clinically inapparent tumor that is not palpable
T1a – Tumor incidental histological finding in 5% or less of tissue resected
T1b – Tumor incidental histological finding in more than 5% of tissue resected
T1c – Tumor identified by needle biopsy found in one or both sides, but not palpable
T2 – Tumor is palpable and confined within prostate
T2a – Tumor involves one-half of one side or less
T2b – Tumor involves one-half of one side but not both sides
T2c – Tumor involves both sides
T3 – Extraprostatic tumor that is not fixed or does not invade adjacent structures
T3a – Extraprostatic extension (unilateral or bilateral)
T3b – Tumor invades seminal vesicle(s)
T4 – Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic (pT)
T category
T2 – Organ confined
T3 – Extraprostatic extension
T3a – Extraprostatic extension (unilateral / bilateral) or microscopic invasion of bladder neck
T3b – Tumor invades seminal vesicle(s)
T4 – Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
N category
NX – Regional lymph nodes were not assessed
N0 – No positive regional lymph nodes
N1 – Metastasis in regional lymph node(s)
M category
M0 – No distant metastasis
M1 – Distant metastasis
M1a – Nonregional lymph node(s)
M1b – Bone(s)
M1c – Other site(s) with or without bone disease

Table 2: Prognostic stage groups for PrCa, as classified by the American Joint Committee on Cancer.

Adapted from (Buyyounouski et al., 2017).

T	N	M	PSA	Grade group	Stage group
cT1a-c, cT2a	N0	M0	<10 ng/mL	1	I
pT2	N0	M0	<10 ng/mL	1	I
cT1a-c; cT2a	N0	M0	≥ 10, < 20ng/mL	1	IIA
pT2	N0	M0	≥ 10, < 20ng/mL	1	IIA
cT2b-c	N0	M0	< 20ng/mL	1	IIA
T1-2	N0	M0	< 20ng/mL	2	IIB
T1-2	N0	M0	< 20ng/mL	3	IIC
T1-2	N0	M0	< 20ng/mL	4	IIC
T1-2	N0	M0	≥ 20ng/mL	1-4	IIIA
T3-4	N0	M0	Any	1-4	IIIB
Any	N0	M0	Any	5	IIIC
Any	N1	M0	Any	Any	IVA
Any	Any	M1	Any	Any	IVB

According to the European Society for Medical Oncology (ESMO) guidelines, men with PrCa can be stratified into different risk groups to better guide the therapeutic options (Parker et al., 2020). For example, lower TNM scores and a *PSA* value of < 10 ng/mL are considered to have a low risk of PrCa progression, indicating that patients can be put under active surveillance and can refrain from any treatment options until deemed necessary (Adamaki & Zoumpourlis, 2021).

2.4. Therapeutic options

Nowadays, there are several treatment options available to patients diagnosed with PrCa. These options are explained by the provider and are based on several factors such as the stage and grade of the tumor, the age, and the health of the patient. It is important to inform the patients about all available therapies, their associated benefits, and also potential risks that could lead to complications such as sexual dysfunction, urinary problems, or even infertility (Parker et al., 2020) (Table 3).

Table 3: Different therapeutical options for localized and metastatic PrCa. Adapted from (Parker et al., 2020)

Localized disease		
Low-risk group	Intermediate-risk group	High-risk group

Active surveillance or watchful waiting (<i>recommendation</i>). Prostatectomy, radiotherapy (<i>options</i>).	Prostatectomy, radiotherapy (<i>recommendation</i>). Radiotherapy + ADT (<i>4-6 months</i>).	Prostatectomy, radiotherapy (<i>recommendation</i>). Radiotherapy + ADT (<i>18-36 months</i>).
Metastatic disease		
Hormone-sensitive PrCa (HSPC)	Castration-resistant PrCa (CRPC)	Non-metastatic castration-resistant
ADT (<i>degarelix / relugolix</i>) + AR signaling inhibitors (<i>enzalutamide / abiraterone</i>). ADT + chemotherapy (<i>docetaxel</i>).	ADT + AR inhibitors (<i>abiraterone / enzalutamide</i>) + chemotherapy (<i>cabazitaxel</i>). Immunotherapy (<i>pembrolizumab</i>). PARP inhibitors for DDR mutations (<i>olaparib, rucaparib</i>).	AR inhibitors (<i>darolutamide / apalutamide</i>).

2.4.1. Localized disease

Currently, there are several treatment options available for patients with localized PrCa, which is defined as having no regional lymph nodes with cancer cells or distant metastasis (Litwin & Tan, 2017). For patients in the low-risk group, the recommendation is to follow watchful waiting or active surveillance, although radiotherapy or surgery may also be options (Parker et al., 2020). Watchful waiting involves monitoring the disease without using immediate treatment, while active surveillance entails tracking the cancer's progress using PSA testing, DRE, or several biopsies (Litwin & Tan, 2017). For those in the intermediate or high-risk groups, active surveillance is not recommended. Instead, prostatectomy and radiotherapy are the available approaches, with patients being informed about the benefits and drawbacks of each one (Parker et al., 2020). In fact, the ProtecT trial compared patients who received radiotherapy or prostatectomy and found no significant difference in the overall quality of life up to 5 years between the two treatment groups (Mottet et al., 2021). However, the outcomes of receiving these treatments differ, with patients who received radiotherapy reporting worse bowel function after 6 months, while those who underwent prostatectomy experienced the greatest impact on sexual and urinary functions (Donovan et al., 2016).

Radical prostatectomy is a surgery where the entire prostate gland is removed. It can be performed through open surgery or laparoscopy (Sekhoacha et al., 2022). Currently, laparoscopic radical prostatectomy is the most commonly used method as it is less invasive and allows the surgeon to view the organ (Lein et al., 2006). This procedure is usually recommended for patients under 70 years old, without metastasis, and with a life

expectancy of over 10 years (Sekhoacha et al., 2022). Common side effects of this treatment are incontinence and sexual dysfunction (Militaru et al., 2023). After the surgery, PSA levels should be assessed to evaluate the presence of residual malignant cells, with PSA levels expected to be <0.1 ng / mL. If PSA levels remain persistent or even rising, men can still be treated with radiotherapy (Heidenreich et al., 2014). The two main uses of radiotherapy for PrCa treatment are brachytherapy or external beam radiation. Brachytherapy, mostly used for lower stages of PrCa, uses radiation placed directly into the prostate (Dutta et al., 2018), whereas, the external beam radiation mostly focuses on the tumor volume to eliminate the malignant cells (Podder et al., 2018). Similar to a radical prostatectomy, radiation therapy can also cause sexual, urinary and bowel problems (Militaru et al., 2023).

In addition to radiation therapy, androgen deprivation therapy (ADT) should also be considered for patients with intermediate or high-risk disease (Parker et al., 2020). The basis of ADT consists in the use of luteinizing hormone-releasing hormone (LHRH) agonists or antagonists in order to reduce the androgen amount produced in the testicles. The blockade can be surgical or chemical since PrCa is a hormone dependent cancer (Militaru et al., 2023). The first approach consists of blocking androgen production in the testicles, while in the latter option, an androgen receptor (AR) inhibitor is administered to block the AR pathway, therefore preventing PrCa growth (Militaru et al., 2023; Sekhoacha et al., 2022). ADT, however, also has its side effects, namely sexual dysfunction, hot flashes, cardiac problems, and cognitive impairment (Litwin & Tan, 2017). For patients with intermediate-risk disease, the combined treatment with ADT and radiotherapy lasts for around 4-6 months, while for those belonging to the high-risk group, ADT should be used for longer periods, typically between 18-36 months (Militaru et al., 2023; Parker et al., 2020).

2.4.2. Metastatic disease

The first-line of treatment for metastatic PrCa is using ADT (Litwin & Tan, 2017). This treatment is recommended for metastatic hormone-sensitive PrCa (mHSPC) (Heidenreich et al., 2013), often in combination with AR signaling inhibitors, such as abiraterone or enzalutamide to block the synthesis of androgens, or antagonize the function of the receptor (Harris et al., 2022; Parker et al., 2020). Abiraterone primarily targets the enzyme CYP17A, which is involved in testosterone production. On the other hand, enzalutamide is an AR antagonist that acts by preventing androgens such as testosterone and 5 α -dihydrotestosterone (DHT) from binding to the AR (Sekhoacha et al., 2022).

ADT can also be combined with chemotherapy, with docetaxel being the most commonly used agent in this context (Parker et al., 2020). This drug directly targets the microtubules, inhibiting the function of β -tubulin during mitosis, leading to deregulation of cell division (Sekhoacha et al., 2022). Patients with mHSPC often respond to ADT therapy during the early stages of the disease, which leads to a significantly prolonged progression-free survival. In fact, the length of response to ADT is considered a prognostic factor, since a longer response to this first-line treatment is associated with better overall survival rates compared to a shorter response (Adamaki & Zoumpourlis, 2021). However, in many cases, PrCa becomes unresponsive to hormone therapy and progresses into metastatic castration-resistant PrCa (mCRPC) (Schatten, 2018). This mainly occurs due to the cancer cells' ability to activate the AR to bypass ADT (Adamaki & Zoumpourlis, 2021). According to the ESMO guidelines, docetaxel, abiraterone, and enzalutamide are all recommended treatments for men with mCRPC. Moreover, these three drugs, as well as cabazitaxel, a chemotherapy drug, are also viable options following therapy with docetaxel (Parker et al., 2020). Additionally, for non-metastatic castration-resistant PrCa, the US Food and Drug Administration (FDA) has approved the use of two AR inhibitors, darolutamide and apalutamide, to be used in combination with ADT (Adamaki & Zoumpourlis, 2021).

More recently, targeted therapies have also emerged as treatment options for mCRPC patients. The immunotherapy drug pembrolizumab, which is a *PD-1* inhibitor, has been approved for patients with mismatch repair (MMR) deficiency, since it was demonstrated that alterations in this pathway increase the sensitivity to these agents (Brandao et al., 2020; Vietri et al., 2021). Poly (ADP-ribose) polymerase (*PARP*) inhibitors, like olaparib or rucaparib, are also options, to be used in patients harboring somatic or germline mutations in the DNA damage response (DDR) genes such as Breast Cancer Gene 1 and 2 (*BRCA1/2*) (Robinson et al., 2015).

3. Genetic contributions to prostate cancer

3.1. Somatic alterations

Somatic alterations in genes belonging to the *ETS* transcription factors family, particularly the fusion between the transmembrane protease serine 2 (*TMPRSS2*) and the *ETS*-related gene (*ERG*), are often detected in a percentage of high-grade PIN lesions close to carcinomas (De Marzo et al., 2016). In localized PrCa, this gene fusion is the most common, occurring in approximately 50% of all patients (Sandhu et al., 2021). The loss of

phosphatase and tensin homolog (*PTEN*), common in intraductal carcinomas (De Marzo et al., 2016), and mutations in tumor protein 53 (*TP53*) are found in 10-20% of localized cancer cases (Sandhu et al., 2021). Lastly, overexpression of myelocytomatosis oncogene (*MYC*) and a reduction in NK3 homeobox 1 (*NKX3.1*) expression have been independently associated with PrCa initiation (Sekhoacha et al., 2022).

Once PrCa reaches its metastatic state, it can quickly progress from castration-sensitive to castration-resistant PrCa, with somatic alterations occurring during this process. For instance, mutations in the *PTEN* and the *TP53* genes increase to over 40% (Robinson et al., 2015) and retinoblastoma 1 (*RB1*) loss is also a frequent event associated with progression to castration-resistant cancer (Beltran et al., 2016). Amplifications in the AR are also associated with late-stage disease, and increased resistance to ADT (Coutinho et al., 2016). Additionally, amplifications of other oncogenes such phosphoinositide 3-kinase (*PI3K*), protein kinase B (*Akt*), as well as alterations in tumor suppressor genes including *BRCA1/2*, have also been reported (Carver, 2011; Robinson et al., 2015).

3.2. Germline alterations

PrCa is well-established as having a strong familial component, with 10-20% of patients reporting a family history of the disease (Cheng et al., 2019). Familial and hereditary PrCa (HPC) are both associated with an increased risk of developing the disease, but while familial PrCa refers to cases that cluster within families, HPC, which only accounts for about 3-5% of all cases [28], refers to families with a strong genetic predisposition to PrCa. HPC can therefore be considered a subset of familial PrCa, and its criteria include the presence of PrCa in three consecutive generations, at least three first-degree relatives diagnosed with this disease, or having two or more cases of PrCa diagnosed before the age of 55 years of age (Brandao et al., 2020; Ni Raghallaigh & Eeles, 2022).

In familial PrCa, as well as in HPC, patients are eligible for germline genetic testing (Heidegger et al., 2019; Vietri et al., 2021), nevertheless, different entities have their own specific criteria and guidelines for germline genetic testing. The NCCN, the EAU and ESMO all have specific guidelines for germline genetic testing. According to the NCCN, the criteria for germline genetic testing in PrCa patients is any of the following: having two or three family members affected with PrCa before 60 years of age, high-risk or very high-risk of regional or metastatic PrCa, Ashkenazi Jewish family background, or previous history of breast, pancreatic, colorectal, melanoma, gastric, small intestinal, biliary tract, upper tract

urothelial cancers or glioblastoma (National Comprehensive Cancer Network, 2024a). Meanwhile, the EAU recommends germline testing after genetic counselling, for those fulfilling any of the following criteria: metastatic PrCa, one or more relatives diagnosed before the age of 60 years old, a family member who died from PrCa, or having several cancers on the same side of the family (Mottet et al., 2021). Lastly, ESMO recommends men to be tested for germline mutations when they have two or more blood relatives on the same side of the family diagnosed with any of the following cancers: prostate, melanoma, breast, sarcoma, colorectal, endometrial, brain, ovarian, pancreatic, kidney or thyroid (Parker et al., 2020).

Patients undergoing genetic testing for germline genetic mutations are assessed for specific genes that play a role in the development of PrCa, including those involved in the DDR pathway (National Comprehensive Cancer Network, 2024a; Parker et al., 2020; Vietri et al., 2021). *BRCA1* and *BRCA2* are tumor suppressor genes located on chromosomes 17 and 13, respectively (Zhen et al., 2018). These genes encode proteins that have an essential role in the homologous recombination pathway, which is responsible for the repair of DNA double-strand breaks in response to DNA damage (Yoshida & Miki, 2004). Given their critical role in genome protection, deleterious inherited alterations in these genes can predispose individuals to several malignancies (Brandao et al., 2020; Sekhoacha et al., 2022). Although germline mutations in the *BRCA* genes are primarily associated with Hereditary Breast and Ovarian Cancer syndrome, they also increase the risk of developing PrCa (Zhen et al., 2018). In men with germline mutations in *BRCA1/2*, the risk increases to around 1.8-3.8-fold for *BRCA1* and 4.6-8.6-fold for *BRCA2*, in patients under younger than 65 years old (Heidegger et al., 2019; Vietri et al., 2021). Furthermore, these mutations lead to more aggressive disease, reflected by a higher Gleason score, presence of metastasis at diagnosis, and worse overall survival (Heidegger et al., 2019).

Ataxia-Telangiectasia Mutated (*ATM*), Partner and localizer of *BRCA2* (*PALB2*), and Checkpoint kinase 2 (*CHEK2*), located on chromosomes 11, 16, and 22, respectively (Phan & Rezaeian, 2021; Wu et al., 2020; Zhen et al., 2018) are also tumor suppressor genes that belong to the DDR signaling pathway which have been associated with HPC. *ATM* is a tumor suppressor gene essential in cell apoptosis, cell cycle arrest and DNA damage repair (Phan & Rezaeian, 2021). *ATM* germline mutation carriers have an increased risk for development of HPC, in addition to other cancer types, such as breast, skin, colorectal, pancreatic, or gastric cancer (Heidegger et al., 2019; Vietri et al., 2021). As for *PALB2*, it acts within the *BRCA* complex by aiding in homologous recombination and is specifically associated with breast and pancreatic cancer. Germline mutations in *PALB2* are rare, having been identified in around 0.4% of HPC patients (Vietri et al., 2021). Lastly, *CHEK2*

plays an important role in regulating TP53 (Heidegger et al., 2019; Zhen et al., 2018). Besides PrCa, mutations in *CHEK2* are associated to the Li-Fraumeni syndrome, breast, ovarian, thyroid, kidney, renal, and colon cancers (Heidegger et al., 2019; Ni Raghallaigh & Eeles, 2022; Vietri et al., 2021; Zhen et al., 2018). In PrCa, germline mutations of this gene compose an overall risk of 1.9-3.3% (Vietri et al., 2021).

Homeobox B13 (*HOXB13*), located on chromosome 17 (Zhen et al., 2018) is another gene associated with an increased risk for HPC (Brandao et al., 2020). It is important in embryogenesis and in regulating some *AR* target genes such as *PSA* (Norris et al., 2009; Zhen et al., 2018). Patients with a specific missense mutation, G84E, have an increased risk in early-onset and HPC development (Heidegger et al., 2019).

Besides the homologous recombination pathway, the MMR pathway plays a vital role in preserving genomic integrity, specially by guaranteeing genetic stability post DNA replication (Lynch et al., 2015). Deleterious variants in one of the four MMR genes, MutL homolog 1 (*MLH1*), MutS homolog 2 (*MSH2*), MutS homolog 6 (*MSH6*) and *PMS1* homolog 2 (*PMS2*), located on chromosomes 3p21.3, 2p21, 2p16 and 7p22.2, respectively (Pecina-Slaus et al., 2020), are commonly associated with Lynch Syndrome (LS). LS is an autosomal dominant disease in which the MMR system is defective, resulting in increased microsatellite instability and a higher likelihood of replication errors (Lynch et al., 2015; Ten Broeke et al., 2018). This condition predisposes individuals to several malignancies, with colorectal and endometrial cancers being the most common (Pecina-Slaus et al., 2020). Additionally, MMR genes have been linked to an increased risk of HPC, with the highest risk observed among *MSH2* variant carriers (Vietri et al., 2021; Zhen et al., 2018).

Recently, other genes have been proposed as novel predisposition genes for HPC such as Nijmegen Breakage Syndrome 1 (*NBS1*) and *BRCA1*-interacting protein C-terminal helicase 1 (*BRIP1*) (Brandao et al., 2020). *NBS1*, located on chromosome 8q21, is a tumor suppressor gene involved in the DNA double-stranded damage repair system (Cybulski et al., 2004; Zhen et al., 2018). It is part of a complex associated with *BRCA1*, aiding in the homologous recombination in response to DNA damage (Cybulski et al., 2004). Mutations in the *NBS1* gene are responsible for an autosomal recessive disorder in which there is an increased predisposition to develop cancer, mostly of lymphoid origin (Komatsu, 2016). With regards to PrCa, the most commonly reported mutation found in these patients is a deletion in exon 6 (657del5), particularly found in men with a family history of PrCa (Cybulski et al., 2004). Similar to *NBS1*, *BRIP1* is also an important homologous recombination gene, together with *BRCA1* (Zhen et al., 2018). This tumor suppressor gene, located on chromosome 17q22 has also been associated with Fanconi anemia, a disease characterized by chromosomal instability and early tendency for cancer development

(Mosedale et al., 2005). Ramus *et al.* associated stop-gain mutations in *BRIP1* to an augmented risk of epithelial ovarian cancer in American populations (Ramus et al., 2015). In PrCa, the *BRIP1* loss-of-function mutation c.2392C>T, previously linked to breast cancer, was also found in two PrCa families by Leongamornlert and colleagues (Leongamornlert et al., 2014).

Nonetheless, the percentage of PrCa that can be attributed to hereditary factors is still very scarce, only between 5%-15%, rendering the need for discovering new genes that could explain the remaining familial/hereditary cases (Vietri et al., 2021). In this context, the *Prune* Homolog 2 with *BCH* domain (*PRUNE2*) gene was recently highlighted by our research group as a novel PrCa predisposition gene. Cardoso *et al.* studied 462 previously recruited patients fulfilling one or both of the following criteria: diagnosis of PrCa before the age of 56 and/or familial/hereditary PrCa, where one or more family members were diagnosed before the age of 66. Whole-exome sequencing (WES) was initially performed on 96 patients from 45 families. In this analysis, *PRUNE2* emerged as a strong candidate, and further validation through targeted next-generation sequencing (NGS) in the whole series revealed *PRUNE2* to be the most frequently mutated gene, with pathogenic/likely pathogenic variants found in 2.8% of the HPC patients (13/462). Therefore, *PRUNE2* was proposed as a novel candidate biomarker for PrCa predisposition (Cardoso et al., 2023).

4. *PRUNE2*/PCA3 axis

4.1. *PRUNE2*

PRUNE2, initially called *BMCC1* (*BCH motif*-containing molecule at the carboxyl terminal region 1), was first described by Machida *et al.* as a novel gene with proapoptotic functions. It is particularly expressed in neuroblastomas with a good prognosis (Machida et al., 2006). This gene is a tumor suppressor located on chromosome 9q21.2. It is a member of the B-cell CLL/lymphoma 2 (*Bcl-2*) and adenovirus E1B 19 kilodalton (kDa) family (Martin et al., 2023), comprising of several anti-apoptotic proteins that interact with pro-apoptotic proteins to either inhibit or promote numerous key processes related to apoptosis (Liu et al., 2004). The *PRUNE2* gene possesses a *BCH* domain at the carboxyl-terminus, also called the *BNIP2* (*Bcl-2* / adenovirus E1B 19 kDa-interacting protein 2) and *Cdc42GAP* (Cell division control protein 42 homolog GTPase-activating protein) homology domain (Machida et al., 2006). Proteins containing this domain have previously been involved in multiple

signaling pathways such as apoptosis promotion, cell transformation inhibition, and molecular activation and cell migration by interacting with molecules such as Rho and Ras from the GTPase family, GTPase-activating proteins (GAPs), and guanine nucleotide exchange factors (GEFs) (Pan & Low, 2012).

PRUNE2 has 14 transcripts obtained via alternative splicing, and its canonical form (ENST00000376718) possesses 19 exons and 3088 amino acids (aa) (Martin. et al., 2023). Half of those transcripts produce proteins with molecular weights ranging from 30 kDa to 340 kDa (Lonsdale et al., 2013; UniProt, 2023) and are induced during the G1 phase of the cell cycle (Figure 6) (Machida et al., 2006).

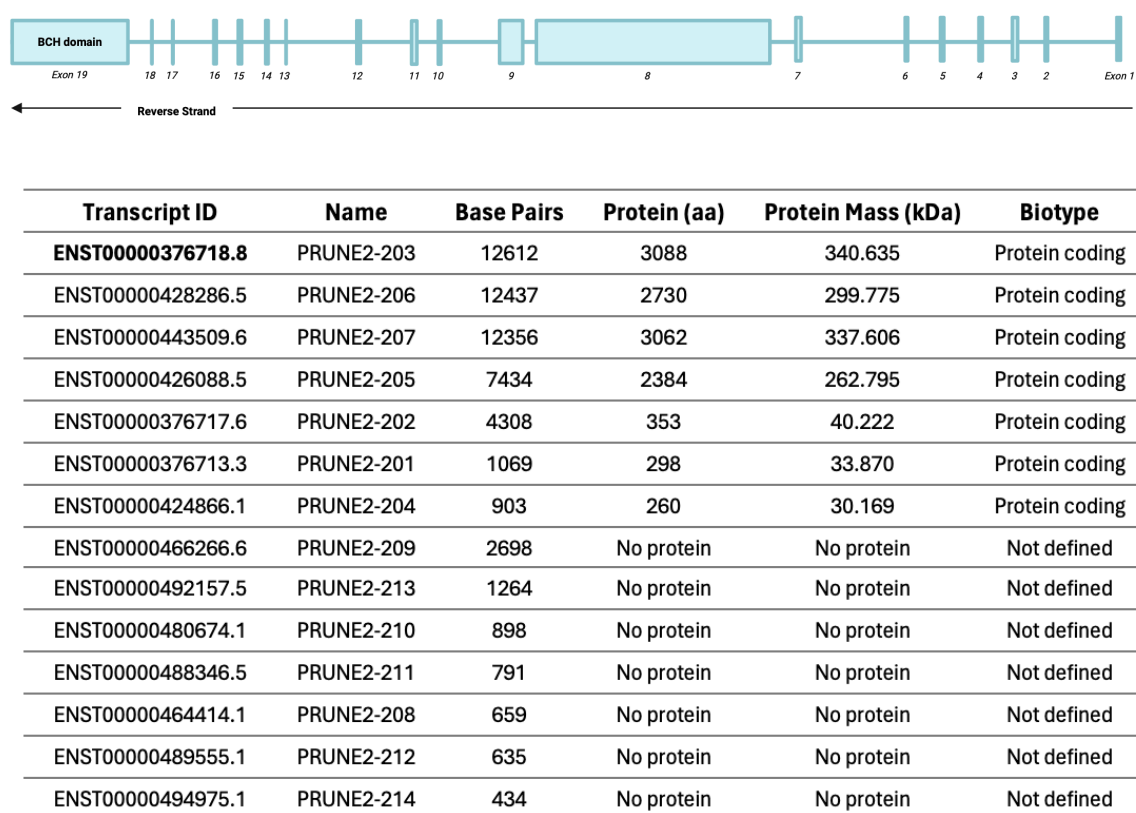


Figure 6: Structure of *PRUNE2* and its 14 transcripts, obtained via alternative splicing. The canonical form of *PRUNE2* contains 19 exons and a BCH domain at the C-terminal. Half of *PRUNE2*'s transcripts produce proteins. Adapted from (Lonsdale et al., 2013) and (Martin. et al., 2023).

In PrCa, the tumor suppressor role of *PRUNE2* is interconnected with a long noncoding RNA (lncRNA) called prostate cancer antigen 3 (*PCA3*), an axis not observed in any other cancer types, since *PCA3* is prostate tissue-specific (Ferreira. et al., 2012). *PCA3* effectively downregulates *PRUNE2* expression levels via an RNA editing mechanism mediated by ADAR proteins (Salameh et al., 2015). Regarding cellular alterations, the

inhibition of *PRUNE2* or the overexpression of *PCA3* has been linked to increased cell proliferation, adhesion, and migration. Alternatively, the silencing of *PCA3* or overexpression of *PRUNE2* has been shown to have the opposite effects on cell proliferation, by decreasing cell viability and migration (Salameh et al., 2015). This contrasting function led Lauer *et. al* to hypothesize the existence of an axis between *PRUNE2* and *PCA3*, where the latter acts as an oncogene by downregulating the expression of the tumor suppressor gene *PRUNE2* (Lauer et al., 2023).

4.2. PCA3

PCA3 is lodged in intron 6 of *PRUNE2* and is transcribed in an antisense direction (Salameh et al., 2015). It is located in chromosome 9q21-22, with a length of around 25 kb, and has 4 exons (Figure 7) (Bussemakers et al., 1999). *PCA3*'s expression seems to be restricted to prostate cells and overexpressed in prostatic tumors compared to normal prostate tissues (Bussemakers et al., 1999).

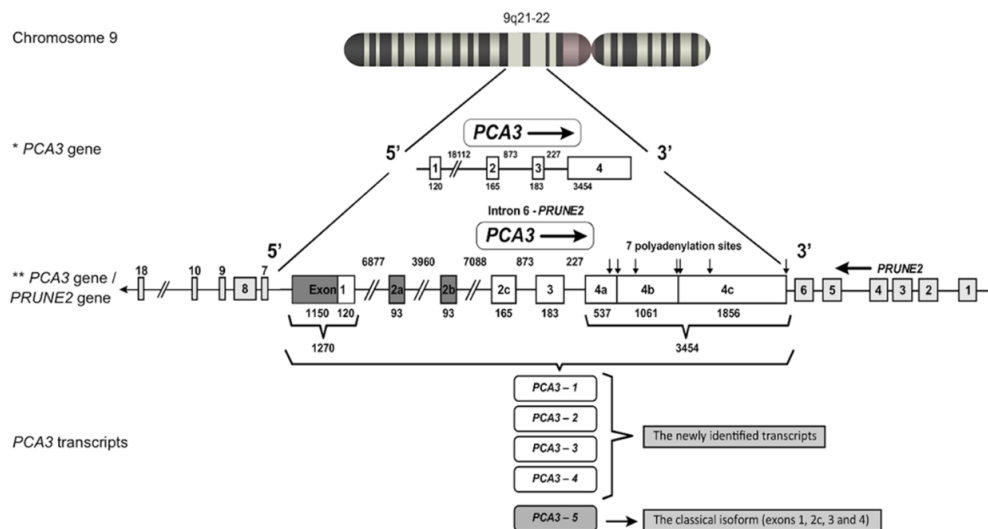


Figure 7: Chromosomal location and structure of the *PCA3* lncRNA. *PCA3*, located on chromosome 9, was initially thought to have only 4 exons, until the discovery of two additional exons. Its main isoform, *PCA3*-5, contains the exons 1, 3, 4a and 4b. Adapted from (Lemos et al., 2019).

Similar to other lncRNAs that are known to play crucial roles in tumor development and progression by regulating gene expression and the cell cycle, *PCA3* is one of the most studied lncRNAs in PrCa, due to its tissue specificity (Lauer et al., 2023). Consequently, it has been approved as a complementary biomarker in diagnosing PrCa in the European

Union and the United States since 2012 (Salameh et al., 2015). *PCA3* levels do not depend on patient age, trauma, inflammation, or prior biopsies (Lemos et al., 2019), which led to the FDA approval of the Progenesa™ *PCA3* test. This test, which measures the concentration of *PCA3* and *PSA* RNA in urine samples collected after a DRE is also available in Europe, in combination with patient history, in men older than 50 years old who have had negative biopsies to help determine if a repeated biopsy is necessary (Gunelli et al., 2021).

Studies have shown that silencing *PCA3* reduces cell viability, growth, and survival, and promotes apoptosis in PrCa cells (Ferreira. et al., 2012). *PCA3* has been shown to contribute to carcinogenesis, by regulating several mechanisms, such as DNA repair, promoter looping, antisense gene silencing and/or transcriptional regulation, making it a promising candidate as a cancer biomarker (Salameh et al., 2015). Furthermore, *PCA3* was shown to be upregulated in cells treated with an *AR* agonist (DHT), and downregulated upon treatment with an *AR* antagonist (flutamide), effectively linking this lncRNA with the function of the *AR* pathway (Figure 8) (Lemos et al., 2019).

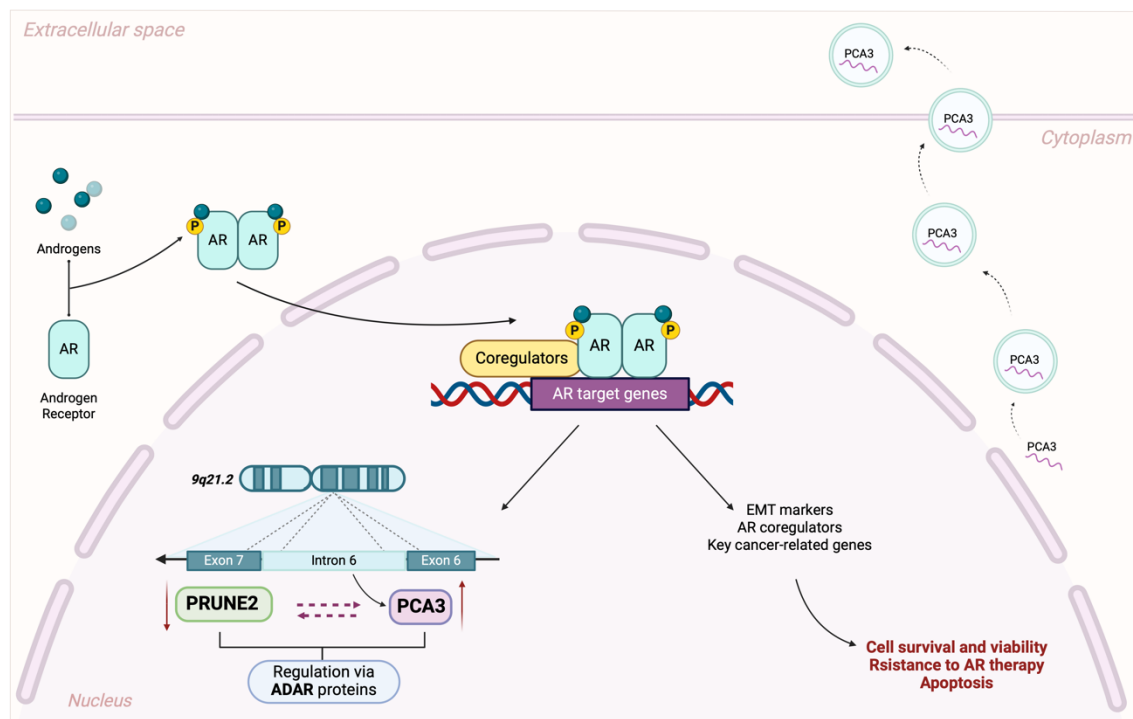


Figure 8: Overview of *PCA3*'s functions in the cell and relationship with *AR*. *PCA3* is bound to *PRUNE2* pre-mRNA and regulated via ADAR proteins. It can also connect to *AR* and alter the expression of *AR* coregulators, EMT markers and other cancer-related genes to promote cell survival. Created with BioRender and modified from (Lemos et al., 2019).

4.3. *PRUNE2/PCA3/AR* triad

Previous research has shown that the regulation of the *PRUNE2/PCA3* axis seems to be dependent on *AR* activation (Salameh et al., 2015). Additionally, Özgür *et al.* also demonstrated that inhibiting *AR* with the androgen antagonist enzalutamide reduced *PCA3* levels in the tumorigenic PrCa cell line LNCaP (Ozgur et al., 2017). Interestingly, *AR* also seem interconnected with the *PI3K/Akt* signaling pathway. *AR*'s signaling increases upon targeted *PI3K/Akt* drug administration, whereas the opposite is also observed, with the activation of the *PI3K/Akt* pathways as a consequence of *AR*-targeted therapy (Shorning et al., 2020). Furthermore, prior studies have tested the combination of different drugs, such as *PI3K* inhibitor BEZ235 and enzalutamide (Carver, 2011), as well as the *Akt* inhibitors capivasertib (AZD363) and bicalutamide (Thomas et al., 2013), and reported more efficiency in reducing the number of cancerous cells, as opposed to monotherapy. Given this, the lack of studies associating *PRUNE2* with oncogenic signaling pathways such as *PI3K/Akt* in PrCa emphasizes the relevance of investigating the role of *PRUNE2* in the *PRUNE2/PCA3/AR* triad and specific oncogenic signaling pathways in PrCa development.

4.4. *PRUNE2* in other cancer types

Since its first report, several other studies have focused on *PRUNE2*'s function in multiple cancer types. *PRUNE2* is highly expressed not only in nerve tissues (Islam et al., 2019; Iwama et al., 2011; Li et al., 2011; Tatsumi et al., 2015) but also in several types of cancer such as colorectal cancer (Li et al., 2022), leiomyosarcoma (Zhao et al., 2013), squamous cell carcinoma (Zheng et al., 2022), parathyroid carcinoma (Marini et al., 2022; Storvall. et al., 2023), and PrCa (Cardoso et al., 2023; Lauer et al., 2023; Salameh et al., 2015). Machida and colleagues noted high expression levels in tissues related to the nervous system such as the spinal cord, brain, and cerebellum (Machida et al., 2006). Higher levels of this gene were also associated with a younger age and more favorable stages of neuroblastomas. Neuroblastomas with better prognosis also showed higher expression of *PRUNE2* in the cell cytoplasm (Machida et al., 2006).

Furthermore, *PRUNE2*'s contribution to mediate proapoptotic molecules such as *FOXO3a* (forkhead box O3 protein transcription factor), *Akt*, *ATM*, and *BIM* (Bcl-2 Interacting Mediator of cell death) in neuroblastomas was also investigated (Tatsumi et al., 2015). Tatsumi *et al.* administered the DNA-damaging drug cisplatin to neuroblastoma cells, which caused the induction and overexpression of *PRUNE2*, consequently increasing cell sensitivity to drugs. Upon *PRUNE2*'s inhibition, there was a decrease in the number of cells

experiencing apoptosis (Tatsumi et al., 2015). *PRUNE2*'s overexpression also contributed to mitochondrial apoptosis caused by *Caspase-9* activation, which indicates a negative regulation of the survival pathway through inhibition of *Akt*. As a result, *FOXO3a*, normally inhibited by phosphorylation via *Akt*, became more active, leading to its enhanced proapoptotic activity. Additionally, the inhibition of *Bcl-2* and the phosphorylation of *ATM*, lead to an increase in apoptosis (Figure 9) (Tatsumi et al., 2015).

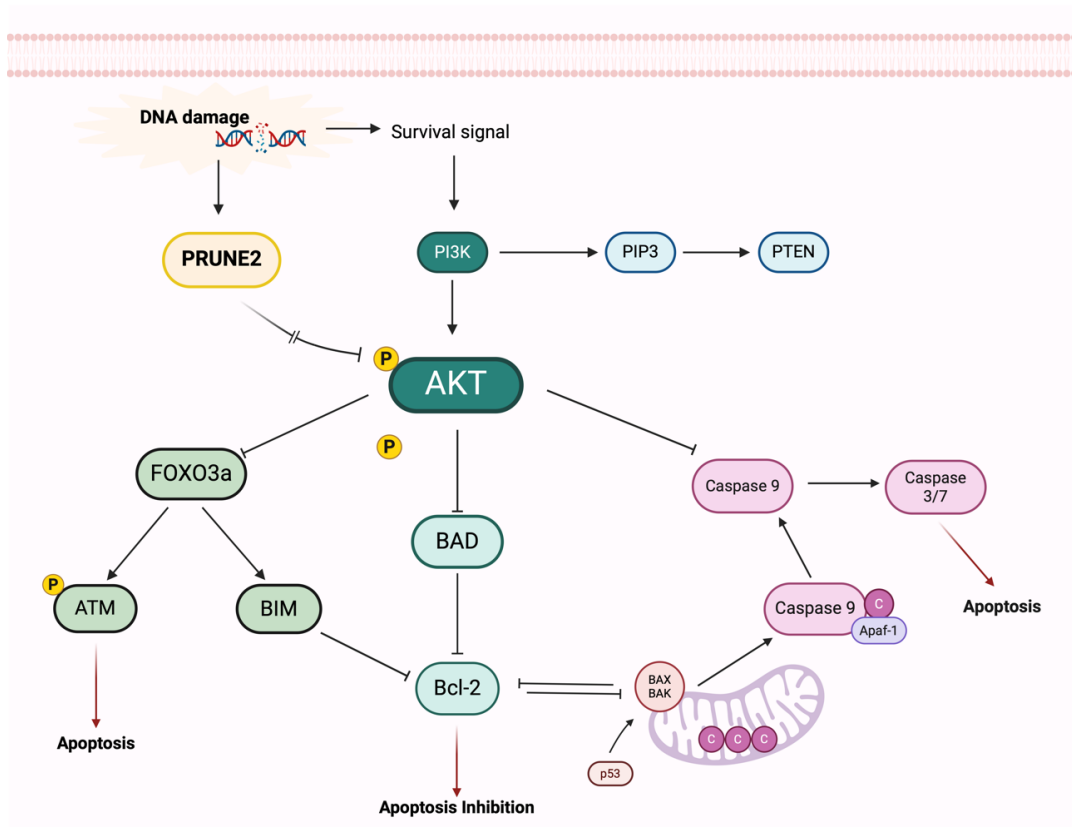


Figure 9: Proposed pathway depicting the interaction between *PRUNE2*, *Akt*, and other apoptosis-related components, in neuroblastomas. *PRUNE2* can interact with *Akt* upon DNA damage and increase the cell sensibility to cisplatin, in neuroblastoma cells. *Akt*, inhibited by *PRUNE2*, caused the activation of *FOXO3a* and an increase in apoptosis. *PRUNE2*'s overexpression also contributed to mitochondrial apoptosis mediated by caspases 3, 7 and 9. Created with BioRender and modified from (Tatsumi et al., 2015)]

In colorectal carcinoma, *PRUNE2* overexpression has been shown to mainly localize to the cytoplasm (Li et al., 2022). Analysis using flow cytometry also revealed that, upon knockdown of *PRUNE2*, the number of cells in phases G0 or G1 of the cell cycle decreased, with a concomitant increase observed in S-phase (Li et al., 2022). *PRUNE2*'s depletion with a short-hairpin RNA (shRNA), or overexpression of *PRUNE2* in colorectal cancer cells has been shown to decrease or increase apoptosis, respectively. Therefore, Li

et al. demonstrated that overexpression of *PRUNE2* promoted apoptosis, by stopping cells from transitioning from G1 to S-phase (Li et al., 2022) and inhibiting the proliferation of malignant cells (Herr. & Debatin., 2001). This gene has also been reported to have a proapoptotic function, with its overexpression being associated with the increased expression of several other proapoptotic proteins, including *Bax*, *Caspase-3* and *Caspase-9*, as well as the decreased expression of antiapoptotic proteins, such as *Bcl-2* and *Cyclin D1* (Li et al., 2022). These proteins are all involved in the mitochondrial cell death pathway, which is activated in response to DNA damage or cellular stress (Herr. & Debatin., 2001).

PRUNE2 has also been described in leiomyosarcomas, where this gene was found to be significantly associated with overall survival and tumor size, leading to the proposal that *PRUNE2* high expression as a good prognostic factor for this cancer (Zhao et al., 2013).

AIMS

Building on the previous finding of pathogenic germline variants in the tumor suppressor gene *PRUNE2* in HPC patients, the aims of this project were to investigate the biological impact of *PRUNE2* on PrCa and to also seeks to explore its involvement in oncogenic signaling pathways to further clarify the role of the *PRUNE2/PCA3/AR* triad in PrCa development and progression. To achieve this, we employed a multidisciplinary approach, integrating various techniques, including computational transcriptomics, siRNA-mediated protein depletion and fluorescence microscopy, divided into four specific objectives:

1. Characterize the transcriptomic profile of *PRUNE2* variant carriers and non-carriers to identify deregulated genes and pathways in carriers of *PRUNE2* germline alterations.
2. Characterize the biological impact and cellular localization of *PRUNE2* in prostate cells, namely the androgen-sensitive PNT1A (non-tumorigenic) and LNCaP (tumorigenic) cell lines.
3. Evaluate the effect of anti-androgen therapy on the androgen-sensitive prostate cell lines using an androgen antagonist (Enzalutamide) and an agonist (DHT), to assess potential differences in their response to androgenic and anti-androgenic treatments following the silencing of *PRUNE2* and *PCA3* silencing.
4. Determine the localization and expression levels of deregulated genes and oncogenic signaling pathways identified in the *PRUNE2* germline variant carriers within the androgen sensitive prostate cell lines to evaluate the impact of *PRUNE2* and *PCA3* silencing in these genes.

MATERIALS AND METHODS

1. Whole-transcriptome sequencing data analysis

Whole-transcriptome (RNA-Seq) data from six HPC patient tumor samples, collected at the Portuguese Oncology Institute of Porto (IPO Porto), was previously generated by our research group. The RNA-Seq analysis workflow typically consists of three main stages: (1) library preparation for sequencing, (2) analysis of the generated sequence reads and gene quantification, and (3) statistical analysis of gene expression and transcriptomic alterations (Ayturk, 2019) (Figure 10). Briefly, for the library preparation, RNA was extracted from biological samples and reverse-transcribed into complementary DNA (cDNA). The resulting cDNA was then fragmented, end-repaired, and adapter-ligated to facilitate sequencing. The libraries were amplified via PCR and subjected to quality control checks, including size selection and quantification. Finally, these libraries were sequenced using a NGS platform (Figure 10A). In the second stage of the RNA-seq workflow, the raw sequence reads underwent quality control and trimming to remove any low-quality reads (Kukurba & Montgomery, 2015). Cleaned FASTQ files were mapped to the reference genome using HISAT2 (Kim et al., 2019), and gene expression was quantified with featureCounts (Liao et al., 2014) (Figure 10B).

In the present study, the RNA-seq analysis was focused exclusively on the third stage, where we employed several computational methods to identify significant transcriptomic changes in *PRUNE2* variant carriers (Figure 10C, D).

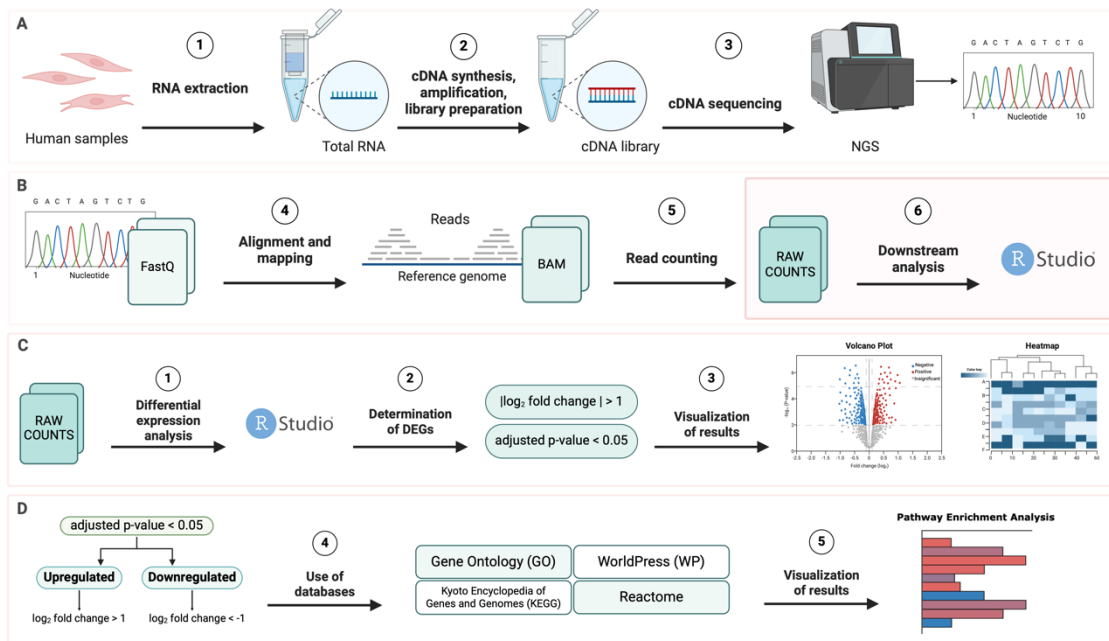


Figure 10: Transcriptomic analysis and RNA-seq workflow. [A] RNA, extracted from human samples (Step 1), is used as a template to synthesize into cDNA (Step 2) and sequenced using NGS (Step 3). [B] The raw

FASTQ sequence is then aligned and mapped to a reference genome (Step 4), followed by raw gene expression count (Step 5) to be analyzed using RStudio (Step 6). [C] Raw gene counts are used for differentially expression analysis which identifies differentially expressed genes (DEGs) (Step 1) that contain a $|\log_2\text{foldchange}| > 1$ and an adjusted p-value < 0.05 (Step 2), and the results are visualized via volcano plot and heatmap (Step 3). [D] The up and down regulated genes are then analyzed using a pathway enrichment analysis, using databases such as GO, KEGG, WP and Reactome (Step 4) and the results visualized using a bar plot (Step 5).

1.1. Differential gene expression (DGE) analysis

The DGE analysis was performed to identify DEGs in *PRUNE2* variant carriers compared to non-carriers. For this analysis, we used RNAseq data from tumor and normal adjacent tissue obtained from the six HPC patients (two *PRUNE2* variant carriers and four non-carriers).

DGE was conducted using DESeq2 (Morganti et al., 2019), a widely used R package, which uses a generalized linear model with a negative binomial distribution to analyze RNA-seq count data. To perform the analysis, raw gene counts of 33,293 genes obtained from featureCounts and the metadata containing two conditions, the sample type - normal or tumor tissues, and *PRUNE2* mutational status - carrier or non-carrier, were imported into RStudio (version 4.3.1) and used as input for DESeq2. Before performing the normalization, counts were pre-filtered to remove genes with low expression across samples and/or with missing values (NA). Despite being an optional step, the removal of genes with lower counts can be useful in decreasing the size of the DESeqDataSet object created the analysis. Notably, in our study, this step reduced the total number of genes to 16,930. Subsequently, normalization was performed to account for differences in sequencing depth and RNA composition between samples, using a median-of-ratios method. The model of DGE analysis included both sample type (normal or tumor tissue) and *PRUNE2* variant carrier status, in order for the model to estimate the effect of *PRUNE2* variants on gene expression while controlling for sample type. Differential expression was estimated using a Wald test integrated into the DESeq2 workflow (Z. et al., 2009). DESeq2 automatically adjusts for multiple testing, using the Benjamini-Hochberg method to control the False Discovery Rate (FDR) (Love et al., 2015).

Genes were considered significantly differentially expressed if they fulfilled the criteria of an adjusted p-value (FDR) < 0.05 , and an absolute $\log_2$ fold change of 1 ($|\log_2\text{fold change}| > 1$). A $\log_2$ fold change of 1 indicates a doubling of expression in a certain gene in the comparison group relative to the control group. Genes with a $\log_2$ fold change > 1 were considered upregulated, while those with a $\log_2$ fold change < -1 were

downregulated. Finally, the results were visualized using a volcano plot and a heatmap to illustrate the patterns of the gene expression changes between the groups. Additionally, the DEGs identified were selected for pathway enrichment analysis to further elucidate the biological pathways deregulated in *PRUNE2* variant carriers.

1.2. Pathway enrichment analysis

Following the DGE analysis, we performed pathway enrichment analysis to investigate deregulated pathways in *PRUNE2* variant carriers compared to non-carriers. This analysis was conducted using the “g:Profiler” toolset, implemented through the R package “gprofiler2”. Within g:Profiler, we used the “g:GOST” functional profiling tool to identify statistically significant biological functions associated with our lists of DEGs (Posit team, 2022).

The enrichment analysis leveraged mainly four comprehensive and complementary datasets: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), WikiPathways (WP), and Reactome (REAC). GO provides annotations to describe the biological functions of genes, non-coding RNAs or proteins. It classifies the gene products according to their cellular components (CC), molecular functions (MF) and roles in biological processes (BP). As of our analysis, GO comprised 41,775 terms: 27,284 for BP, 10,733 for MF and 3,758 for CC (Owens et al., 2019). The KEGG dataset, also focused on cellular processes, connects sets of genes to pathways or complexes based on their known interactions. This database includes information on 110,018 genes and 2,706 pathways (Love et al., 2014). The WP and REAC datasets prioritize information about biological pathways. WP provides insights into 866 human-related pathways, encompassing 36,334 annotations about gene expression products (proteins or RNA), and 7,052 metabolites, resulting in a total of 85,647 interactions (Hong et al., 2020). Lastly, REAC contains annotations of pathways and processes, recording molecular interactions, as well as the effects of diseases and drugs on these pathways (Love et al., 2014). This database covers 2,711 pathways, involving 11,495 proteins and 1,047 drugs (Tsai, 2021).

Pathways with an FDR-adjusted p-value < 0.05 were considered statistically significant. The results were visualized using a bar plot, where significant pathways were divided into upregulated or downregulated clusters, based on whether the genes contributing to its enrichment came from the upregulated or downregulated DEGs list.

2. Cell lines and cell culture

The cell lines chosen for this study were two androgen-sensitive lines derived from the human prostate. The LNCaP cell line, isolated from a metastatic lymph node derived from prostate carcinoma (Castanares et al., 2016), was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). PNT1A, which served as a control, is a non-tumorigenic cell line derived from the prostatic epithelium (Avances et al., 2001), and was purchased via Sigma-Aldrich Corporation (Missouri, USA). Both cell lines were maintained in RPMI 1640 medium, supplemented with 1% Pen Strep and 10% fetal bovine serum. All supplements and culture media were obtained via GIBCO (ThermoFisher Scientific Inc., Massachusetts, USA). Cells were grown in a humidified atmosphere (37°C, 5% CO₂) and regularly tested for *Mycoplasma* contamination via nested PCR (PCR Mycoplasma Detection Set; Clontech Laboratories).

3. Establishment of cellular models

To investigate the functional roles of *PRUNE2* and *PCA3* in PrCa, we proceeded to the establishment of knockdown models using a short interfering RNA (siRNA) for LNCaP and PNT1A cell lines.

3.1. *PRUNE2* and *PCA3* siRNA-mediated depletion

For the creation of cellular models to probe for the functions of *PRUNE2* and *PCA3*, we performed a small interfering RNA (siRNA)-mediated depletion of *PRUNE2* and *PCA3* in both LNCaP and PNT1A cells. siRNAs for *PRUNE2* and *PCA3* were both obtained via GIBCO (ThermoFisher Scientific Inc., Massachusetts, USA, siRNA ID s46087 and n260223, respectively). LNCaP and PNT1A cell lines were transfected with specific siRNAs using adaptations of the protocols available from previous reports (Li et al., 2022; Ozgur et al., 2017). After cells are grown to a confluence of ~100%, they were resuspended into 10 mL RPMI complete medium and 3% of cells were plated per well, in 6-well plates (to achieve a confluence of ~50% the following day). The next day, the medium was removed and replaced with 1 mL Optimem for 1 hour to induce serum starvation and increase siRNA uptake in cells. siRNA reactions were then prepared (Table 4): reaction A is composed of

Optimem and RNAiMAX, while reaction B has Optimem and siRNA *PRUNE2/PCA3*. Control experiments were performed using Optimem only for Reaction B. Both A and B were incubated separately for 5 minutes, followed by an addition of reaction B into A and an incubation for 45 minutes. After 6-8h hours of incubation with siRNAs, 2 mL of RPMI complete medium was added to each well. For initial optimization experiments, siRNA concentration was titrated (25 nM, 50 nM and 100 nM) and samples collected for analysis 48h, 72h, 96h and 120h after siRNA transfection, to determine the optimal concentrations and time points for the *PRUNE2* or *PCA3* depletions.

Table 4: siRNA-mediated protein depletion reactions. Reaction A is composed of RNAiMAX and Optimem, while Reaction B contains Optimem and siRNA in different concentrations. For control cells, reaction B only contains Optimem. The reactions are incubated in separate for 5 minutes, followed by the addition of B into A and an incubation for 45 minutes.

1 6-well plate	Reaction A	Reaction B (control)	Reaction B (25 nM)	Reaction B (50 nM)	Reaction B (100 nM)
RNAiMAX	2 μ L	—	—	—	—
Optimem	248 μ L	250 μ L	247.22 μ L	246.25 μ L	242.5 μ L
siRNA	—	—	1.88 μ L	3.75 μ L	7.5 μ L

PRUNE2 depletion was directly evaluated by Western Blot (WB) and Immunofluorescence (IF) techniques. Due to *PCA3*'s nature as a lnc-RNA, it does not produce protein, therefore, its knockdown was directly validated by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis. Furthermore, we indirectly evaluated *PCA3*'s knockdown by WB and IF by measuring the increase in *PRUNE2* levels. To monitor the depletions by WB and IF, we used a rabbit polyclonal IgG antibody for *PRUNE2*, obtained from ProteinTech (Manchester, UK). For RT-PCR, the *PCA3* primer was purchased from Integrated DNA Technologies Inc. (Iowa, USA, catalogue #Hs.PT.58.25972153.g).

3.2. RNA extraction

RNA extraction was performed in order to validate the depletion of *PCA3* using RT-PCR. For that, two different kits were used: the Qiagen AllPrep DNA/RNA/Protein® Mini Kit (Hilden, Germany), and the Illustra Triple Prep Kit from Cytiva (Danaher Corporation, Washington, D.C., USA).

Transfected and control cells were harvested and stored as pellets at -70°C before the procedure. These were grown in a monolayer in cell-culture T75 flasks, washed with 4 mL Phosphate-Buffered Saline (PBS) (GRiSP, Porto, Portugal), and trypsinized with 2 mL TrypLE™ Express Enzyme (GIBCO) for 10 minutes. Then, we added 8 mL of culture media to the cell suspension, transferred the cells into a 15 mL falcon tube and centrifuged at 12000 rpm for 5 minutes. Then, the culture media was discarded and replaced with 1 mL of new culture media, transferred into a 1.5 mL eppendorf and centrifuged at 300 x g for 5 minutes. The culture media was once again removed, and pellets were stored at -80°C.

When using the Qiagen AllPrep DNA/RNA kit, the pellets were initially flicked and disrupted using 350 µL of Buffer RLT Plus, vortexed, and fully homogenized using a 20-gauge needle in an RNA-free syringe. For DNA collection, the lysate was placed in an AllPrep DNA spin column with a 2 mL collection tube and centrifuged at $\geq 10\,000$ rpm for 30 seconds. The DNA remained in the column, while the RNA was washed into the collection tube. We added 350 µL of 70% ethanol to the flowthrough, mixed it by pipetting, and transferred the entire volume to a RNeasy spin column placed in another collection tube. After centrifuging at $\geq 10\,000$ rpm for 15 seconds, and discarding the flowthrough, we added 700 µL of Buffer RW1 to the column and centrifuged again at the same speed and duration. This was followed by another wash of the column with 500 µL Buffer RPE and another centrifugation at $\geq 10\,000$ rpm for 15 seconds. A second wash with 500 µL of Buffer RPE was performed, followed by a centrifugation at $\geq 10\,000$ rpm for 2 minutes in order to dry the RNA column. Then, the column was transferred to a new collection tube and centrifuged at full speed for 1 minute. Finally, the RNA was eluted from the column into a new 1.5 mL collection tube by using 30-50 µL RNase-free water and centrifuging at $\geq 10\,000$ rpm for 1 minute.

When using the Cytiva Illustra Triple Prep Kit, the pelleted cells were initially washed with 500 µL of PBS and centrifuged at 11 000 rcf for 1 minute. The supernatant was removed, and the cells were disrupted using 350 µL of Lysis buffer type 15 with 3.5 µL of 2-mercaptoethanol (Sigma-Aldrich), resuspended, and fully homogenized using a DNase and RNase-free syringe with a 20-gauge needle. First, a DNA column with a 2 mL collection tube was centrifuged with the lysate at 11 000 rcf for 1 minute. The flowthrough, which contained the RNA, was mixed with 350 µL of pure acetone, placed in an RNA column with a new collection tube and spined at 11 000 rcf for 1 minute. The column, which contained the RNA, was placed in another collection tube and treated to remove residual DNA with a mixture of

90 μ L DNase I, 20 μ L of DNase reaction buffer type 1 and 70 μ L of RNase-free water. This was placed in the center of the column and incubated for 10 minutes, followed by 500 μ L of Wash Buffer type 6 and another centrifugation at 11 000 rcf for 1 minute. Lastly, to elute the RNA, the column was transferred to a new RNase-free 1.5 mL collection tube, followed by an addition of 50 μ L of Elution Buffer type 9 and spin of the column for another minute at 11 000 rcf.

After RNA extraction, samples were quantified using the NanoDrop Microvolume Spectrophotometer (ThermoFisher Scientific Inc., Massachusetts, USA). These were thawed in ice, homogenized by flicking the tubes, and pipetted using 1 μ L of RNA. Concentrations (ng/ μ L) and purity ratios (A260/A280; A260/A230) were recorded and used in cDNA synthesis. The purified RNA was then stored at -80°C for later use.

3.3. Protein extraction

Protein was extracted from transfected cells using the protocol from the Qubit Protein BR Assay Kit (Invitrogen, ThermoFisher Scientific Inc., Massachusetts, USA). Briefly, mixtures were prepared using 2 μ L protein sample with 18 μ L RIPA buffer (dilution of 1:10), for protein samples, 20 μ L Standard 1 or 2 for standards, and 20 μ L RIPA Buffer for blanks. For each of these, we initially added 150 μ L Qubit Protein BR Assay Buffer, followed by 30 μ L Qubit Protein BR Assay Reagent, vortexed each mixture, and incubated them at room temperature for 10 minutes. Afterwards, each tube was read, and its final concentration was multiplied by 10 (to account for the 1:10 dilution step) to obtain the correct protein quantity in each sample. These values were subsequently used in WB assays.

3.4. Western Blot (WB)

PRUNE2 and *PCA3* depletions were monitored by WB. We initially performed a titration to determine the ideal amount of protein detected in each cell line, by running a WB with 10, 20, 30 and 60 μ g of protein. The samples were prepared by mixing the extracted protein, deionized water (ddH₂O), and 5x loading buffer (Tris-HCl, pH 6.8; sodium dodecyl

sulfate (SDS); glycerol, β -mercaptoethanol and bromophenol blue) in a final volume of 22 μ L. Samples were denatured for 5 minutes at 95°C, before loading.

These samples were loaded into a 1.5 mm thick SDS-polyacrylamide gel used for electrophoresis (SDS-PAGE), which is composed of a separating gel (10% acrylamide) and a stacking gel (4% acrylamide), in the quantities found in Table 5. Both of these gels are composed of 40% Acrylamide/Bis (Bio-Rad Laboratories Inc., California, USA), ddH₂O, 10% SDS (in house), TEMED (in house) and APS (20%) (in house). The separating gel also contains 1.5M Tris.HCl (pH 8.8) (Bio-Rad Laboratories Inc), and glycerol (Sigma-Aldrich) while the stacking gel contains 0.5M Tris.HCl (pH 6.8) (Bio-Rad Laboratories Inc).

Table 5: Composition of 4% and 10% acrylamide gels used for SDS-PAGE in WB analysis. Gels are composed of 40% Acrylamide/Bis, 1.5M Tris.HCl (pH 8.8)/ 0.5M Tris.HCl (pH 6.8), 10% SDS, Glycerol, ddH₂O, TEMED and APS (20%)

Solutions (1.5 mm gel)	10% Separating gel	4% Stacking gel
40% Acrylamide/Bis	2.5 mL	250 mL
1.5M Tris.HCl (pH 8.8)	2.5 mL	–
0.5M Tris.HCl (pH 6.8)	–	630 mL
10% SDS	100 mL	25 mL
Glycerol	400 mL	–
ddH ₂ O	4.5 mL	1.59 mL
TEMED	10 mL	5 mL
APS (20%)	50 mL	12.5 mL
TOTAL	~10 mL	~2.5 mL

After preparing the gel using the Bio-Rad WB system, we immersed it in a Tris-Glycine-SDS (TGS) running buffer (composed of 900 mL ddH₂O and 100 mL TGS buffer 10x (Bio-Rad Laboratories Inc.). Samples were then loaded into the gel and ran for around 90 minutes at 120 V. Once the electrophoresis was completed, proteins were transferred from the gel to a nitrocellulose membrane (Advansta Inc., California, USA) with the aid of a Tris-Glycine buffer (TG), consisting of 100 mL TG buffer 10x (Bio-Rad Laboratories Inc.), 700 mL ddH₂O and 200 mL methanol (Sigma Aldrich). The transfer was performed at 100

V for approximately 1 hour on ice, with a magnetic stirrer to reduce the deleterious effects of over-heating.

Following the protein transfer step, the membrane was incubated with agitation for 30-60 minutes with a blocking solution composed of 5% Powder Milk (PM) in PBST (0.05% Tween, Sigma Aldrich). For primary antibody incubations, we used the α -Rb *PRUNE2* antibody (ProteinTech, catalogue #11458-1-AP) diluted at 1:1000 in 1% PM/PBST. For the loading control, we used β -actin (Sigma Aldrich, catalogue #A2228) at a dilution of 1:8000. Primary antibody incubations were either performed for 3hr at room temperature or overnight at 4°C, with agitation. Before incubation with the secondary antibodies, membranes were washed 3X 10min with PBST (0.05% Tween), with agitation. For the secondary antibodies, we used anti-rabbit HRP (1:100 000 dilution) (Santa Cruz Biotechnology, catalogue #sc-2357) or anti-mouse HRP (1:2 500 dilution) (Bio-Rad, catalogue #1706516) in 1% PM/PBST to detect *PRUNE2* or Actin, respectively. Secondary antibody incubations were performed for 1 hour at room temperature with agitation, and subsequently washed for 3X 10min with PBST (0.05% Tween), before adding the Chemiluminescent Substrate kit.

The detection was performed using the SuperSignal West Atto Ultimate Sensitivity Chemiluminescent Substrate kit (ThermoFisher Scientific Inc., Massachusetts, USA), by mixing the Luminol/Enhancer Solution and the Stable Peroxide Solution with PBS (1x) at a dilution of 1:5. The prepared solutions were then evenly distributed across the membrane, which was then placed between a clear plastic inside a cassette, and developed in a darkroom. Lastly, we placed a sheet of autoradiography film on top of the membrane (with varying exposure times), and developed the film using a developer and a fixer. The intensity of the bands was quantified using the ImageJ software (Schindelin et al., 2012). We used the same region of interest (ROI) on each band to measure the pixel density of our target proteins, of β -actin and the background. The measurements were then analyzed on Microsoft Excel, and the background measurements were subtracted from the bands' values. Lastly, the measurements were normalized for the Control sample and creating a relative quantification of protein divided by the loading control.

3.5. Immunofluorescence (IF)

Quantitative IF analysis was performed to validate the depletion of *PRUNE2* or *PCA3* and to visualize *PRUNE2*'s cellular location. In order to accomplish this, sterilized coverslips were initially placed in 6-well plates before cells were plated (Im et al., 2019).

Cells were then plated at a confluence of 3% and allowed to adhere. After cells were transfected and grown until the ideal depletion time point, the RPMI medium was removed from each well and cells were fixed to the coverslip using 4% paraformaldehyde (PFA) (ThermoFisher Scientific Inc.) in PBS for 10 minutes. After fixation, extraction was performed by washing twice for 7 minutes with TBST (0.5% Triton) (Sigma Aldrich) and cells were then incubated in 1X PBS until adding the blocking solution.

To start the IF staining, we incubated coverslips with 50 μ L of IF Blocking solution, composed of PBS with Tween 20 (PBST) + 10% Fetal Bovine Serum (FBS). Coverslips were incubated with IF Blocking solution on parafilm (cell surface facing downwards), in a humidified chamber. After the blocking, we incubated the coverslips with a primary antibody solution (α -Rb *PRUNE2* antibody; ProteinTech, catalogue #11458-1-AP) at a concentration of 1:500. Other assays used the following antibodies: *LAMC2* Ms monoclonal (ABCAM, catalogue #AB210959); *AKT1* + *AKT2* + *AKT3* Rb monoclonal (ABCAM, catalogue #AB179463) and *Bcl-2* Ms monoclonal (ThermoFisher Scientific Inc., catalogue # MA5-11757).

Primary antibody incubations were performed using 50 μ L per coverslip, on parafilm (cell surface facing downwards), in a humidified chamber for 2-3 hours. Coverslips were then washed thrice with PBST (0.05% Tween) for 5 minutes each, and incubated with the secondary antibody solution, containing α -Rb Alexa 568 (ThermoFisher Scientific Inc., catalogue #A-1101) and α -Ms Alexa 488 (ThermoFisher Scientific Inc., catalogue #A-11059) diluted to a concentration of 1:1000 in IF Blocking solution. 50 μ L were used per coverslip, which were incubated for at least 1 hour on parafilm (cell surface facing downwards), in a humidified chamber, protected from light. Finally, cells were washed twice with PBST (0.05% Tween) for 5 minutes, and once with 1X PBS with DAPI (Sigma-Aldrich, catalogue #D9542) (1:50 000 dilution) to stain nuclei. Coverslips were then mounted onto microscope slides using 7 μ L of home-made mounting media (composed of 25mM N-propyl gallate, 10M Glycerol and 20mM Tris.HCl). Excess mounting media was dried off with a paper towel and coverslips were subsequently sealed with nail polish. IF analysis was performed using a BioTek Cytation C10 Confocal Imaging Reader microscope (Agilent Technologies) according to manufacturer instructions. The images were opened on ImageJ, and, using a circular ROI, 30 cells from each image were marked and the pixel density for *PRUNE2* intensity in the nucleus or in the cytoplasm obtained. After a background correction, we calculated the mean and the standard deviation and transported the values into the GraphPad Prism software (version 10.1.1).

3.6. cDNA synthesis

cDNA was synthesized from total RNA extracted from transfected cells using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific Inc., Massachusetts, USA) according to the manufacturer's instructions. Briefly, 1000 ng of each RNA sample were added to a nuclease-free sterile tube, followed by nuclease-free water up to a total volume of 11 μL , and completed with 1 μL of oligo(dT)₁₈ primers. After a spin down and an incubation at 65 °C for 5 minutes, the following components were added per tube: 4 μL 5X Reaction Buffer, 2 μL 10 mM dNTP Mix, 1 μL RiboLock RNase Inhibitor (20 U/ μL) and 1 μL RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/ μL). Components were mixed, centrifuged (spin down), and incubated at 25 °C for 5 minutes, followed by 42 °C for 60 minutes, and lastly at 70 °C for 5 minutes. Subsequently, cDNA was diluted 1:10 with nuclease-free water to allow for multiple future experiments, and stored at -20 °C for later use.

3.7. Quantitative Real-time Reverse Transcriptase PCR (qRT-PCR)

We used the TaqMan technology for the qRT-PCR. This technique adds a probe to the PCR primers, in which this probe contains a quencher molecule at the 3' end and a fluorescent reporter dye at the 5' end. When the cDNA is denatured, both the primers and the TaqMan probe bind to their target sequences, separating the reporter and the quencher, and releasing fluorescence that is detected with each PCR cycle (Medhurst, 2000)

For the qRT-PCR, 4 standards of different concentrations were initially prepared. The standards were prepared in triplicate and in concentrations of 1:5; 1:10; 1:50 and 1:100, using the selected sample and nuclease-free water. For the samples and blanks, we prepared them in duplicate, and started by pipetting 2 μL of cDNA or nuclease-free water into 96-well plates. Meanwhile, for our mixture, we used 10 μL per sample of 2x SensiFAST Probe Lo-ROX Mix, (master mix), obtained from Meridian Bioscience Inc. (Ohio, USA). To these, we added 7 μL of nuclease-free water and 1 μL per sample of the *PCA3* PrimeTime® Std qPCR Assay primer (Integrated DNA Technologies Inc., Iowa, USA), for our lncRNA of interest, or 1 μL per sample of Beta glucuronidase (GUSB) (DQ) Oligo Mix (20x) (Applied Biosystems, ThermoFisher Scientific Inc., Massachusetts, USA) for our housekeeping gene, used as a control. The mixture was homogenized and centrifuged (spin down) before the reaction. Samples were incubated at 50°C for 2 minutes, 95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute, repeated for a total of 40 cycles.

The quantification of the results was obtained with the use of a standard curve built using the previously prepared standards, in order to evaluate the reaction efficiency of our target and reference gene. For the expression analysis, *PCA3* was normalized to the expression of *GUSB* using the comparative cycle threshold (Ct) method. This Ct is the PCR cycle in which the fluorescent signal emitted from the reaction crosses a threshold (Natarajan et al., 2023). The expression value of *PCA3* for each sample was calculated using the formula:

$$2^{-\Delta Ct} = 2^{-(Ct_{\text{target gene}} - Ct_{\text{housekeeping gene (GusB)}})}$$

4. Anti-androgen therapy

To evaluate the effects of androgen therapy on prostatic cells, we started out by performing a titration in wild-type LNCaP and PNT1A cells with different concentrations of Enzalutamide and DHT and at several cellular densities. For this purpose, cells were counted using the Countess 3 automated Cell Counter (ThermoFisher Scientific Inc.), and for both LNCaP and PNT1A cells, 5 000, 10 000 or 15 000 cells were plated per well, in a 96-well plate. After the initial optimization assays, 10 000 cells/well was selected as the being the optimal cell density for subsequent experiments. The concentrations of drugs tested were 1 μ M, 5 μ M, 10 μ M and 20 μ M for Enzalutamide (Selleck Chemicals, Berlin, Germany, catalogue #HY-700002), and 1 nM, 10 nM, 100 nM and 200 nM for DHT (ThermoFisher Scientific Inc.). These were initially incubated at 37°C for 3 days, and in subsequent experiments for 6-12 days in total. After treatment with the Enzalutamide (antagonist) or DHT (agonist), the effects on cell viability, and apoptosis were evaluated using the Calcein AM (Acetoxymethyl ester of calcein; AnaSpec, California, USA) and propidium iodide (PI) (PI; Cytognos, Salamanca, Spain; CYT-PIR-25) assay.

4.1. Calcein AM and PI assay

The Calcein AM and PI assay is a method used to identify live and dead cells. Calcein AM stains the live cells' nuclei in green, while the PI marks the nuclei from dead cells in red. To determine the proportion of live and dead cells, the media from cells growing in 96-well plates was removed, cells were washed with 1X PBS and incubated in a solution

containing Calcein AM (0.5 μ M) and PI (4 μ M), shielded from light. 100 μ L of this solution was added to each well and then incubated at 37°C for 20 minutes (shielded from light). Fluorescent images were acquired using the 10X objective and the total number positive cells for Calcein AM or PI was calculated automatically using the Gen 5 Image Analysis Software, according to manufacturer instructions.

5. Statistical Tests

Statistical analyses were performed using RStudio, and GraphPad Prism software. To evaluate the differences in gene expression levels between groups, we first assessed the normality of the data using the Shapiro-Wilk test. Given that the gene expression data did not meet normality criteria, the non-parametric Wilcoxon rank-sum test was employed to compare two groups. Conversely, as the data from the cellular models satisfied the assumption of normality, unpaired t-tests were used to assess the differences in means between the two groups.

For all statistical analysis, a p-value lower than 0.05, was considered statistically significant.

RESULTS

1. Whole-transcriptome analysis revealed deregulated key signaling pathways in carriers of *PRUNE2* variants

To assess the biological impact of *PRUNE2* variants, we performed whole-transcriptome sequencing of tumor and normal adjacent tissues from six HPC patients, including two variant carriers and four non-carriers. *PRUNE2* expression was significantly downregulated in tumor tissues compared to normal, while the *PCA3* levels showed the opposite trend (Figure 11A). Moreover, *PRUNE2* variant carriers exhibited considerable reduced *PRUNE2* expression levels compared to non-carriers in both sample types (Figure 11B).

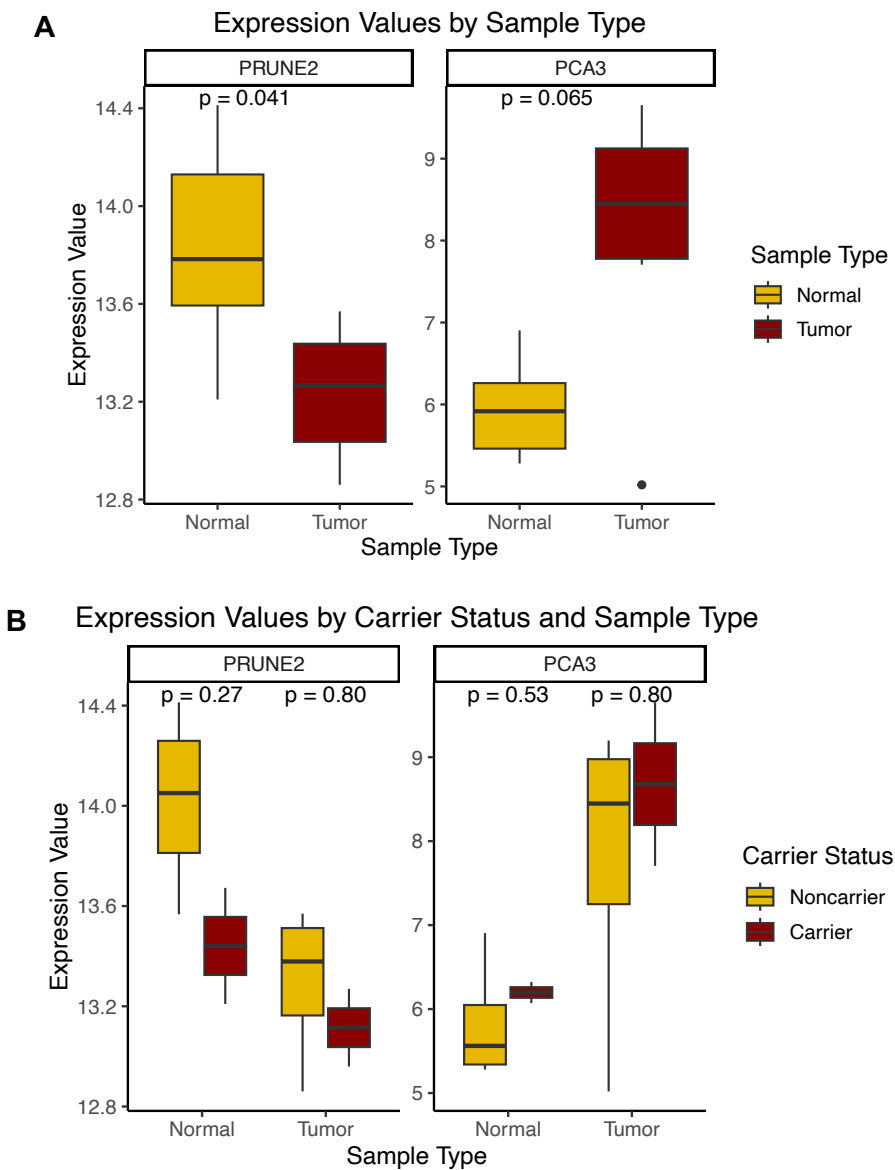


Figure 11: *PRUNE2* and *PCA3* expression levels in HPC patients, evaluated by RNA-seq analysis. Boxplots depict the differences in expression values for *PRUNE2* and *PCA3*. [A] Expression levels ($\log_2$ normalized counts) are compared based on the sample type (normal vs tumor). [B] Expression levels ($\log_2$ normalized counts) are compared between *PRUNE2* variant carriers and non-carriers, separated by sample type.

1.1. Differentially expressed genes (DEGs) in *PRUNE2* variant carriers

To identify DEGs in *PRUNE2* variant carriers, we conducted a comprehensive transcriptomic analysis, comparing the expression profile of all *PRUNE2* carriers and non-carriers, controlling for sample type, to ensure that the observed differential expression was specifically associated with the *PRUNE2* variants, and not differences between sample types (normal and tumor tissues). Considering the stringent criteria of an $|\log_2$ fold change| > 1 and an FDR-adjusted p-value < 0.05 , 86 genes were found significantly deregulated in *PRUNE2* variant carriers compared to non-carriers (Supplementary Table 1, Appendix 1). Of the 86 DEGs, 46 were found downregulated and 40 upregulated in *PRUNE2* variant carriers (Figure 12A).

Comparing the expression profile of the 86 DEGs across conditions, the hierarchical clustering analysis grouped the samples into two main clusters, clearly distinguishing between *PRUNE2* variant carriers and non-carriers, supporting distinct transcriptomic profiles between those groups of patients (Figure 12B).

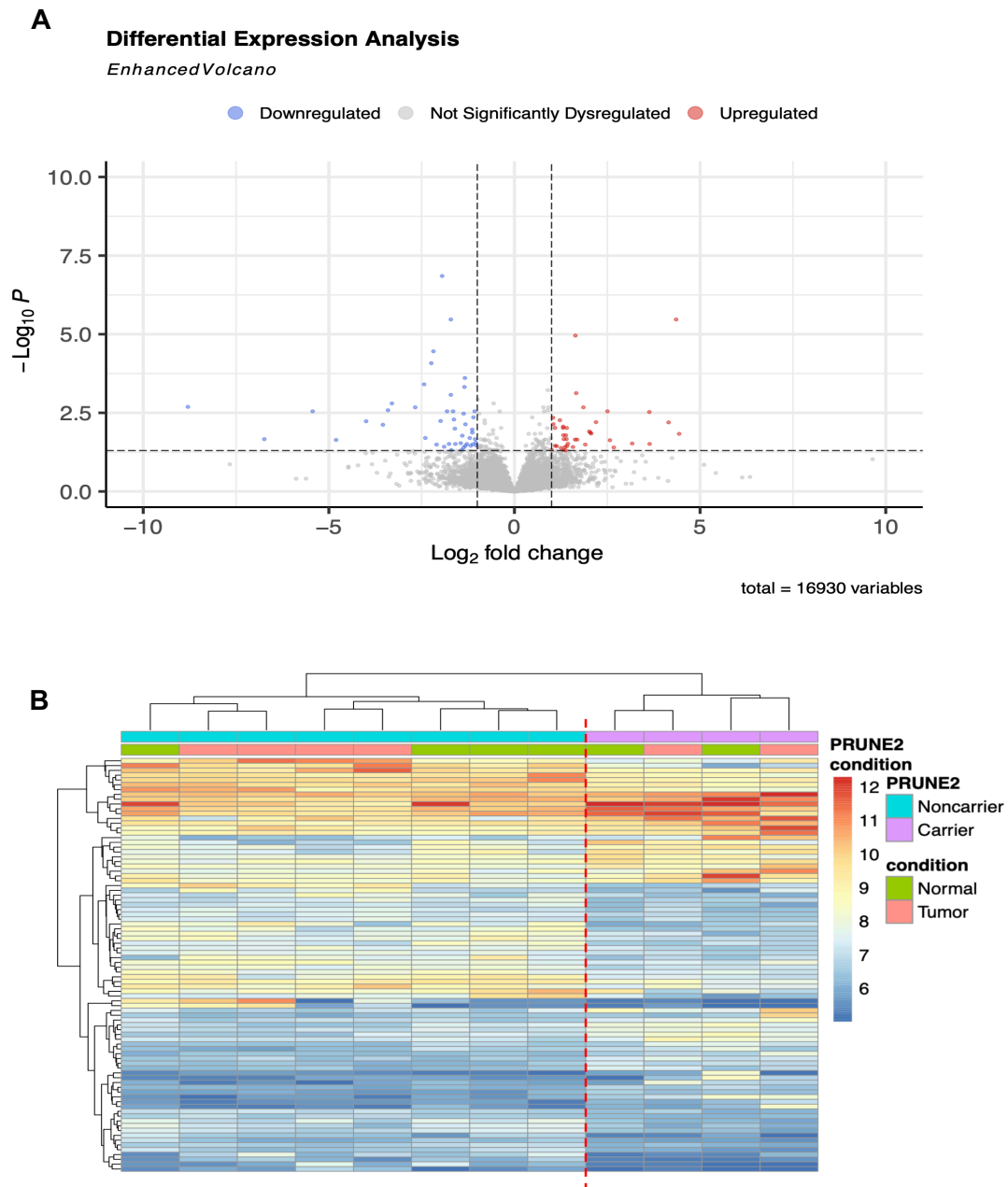


Figure 12: Differential expression of genes in *PRUNE2* variant carriers. [A] Volcano plot showing the deregulated genes in *PRUNE2* variant carriers. In blue, the downregulated genes ($\log_2$ fold change < -1), and in red, the upregulated genes ($\log_2$ fold change > 1). [B] Hierarchical clustering analysis of the 86 DEGs between two *PRUNE2* variant carriers (purple) and four non-carriers (blue).

1.2. Enriched pathways in *PRUNE2* variant carriers

Functional enrichment analysis was performed to identify biological processes, molecular functions, and pathways significantly associated with the 86 DEGs identified in our study. The GO enrichment analysis revealed a significant association (FDR-adjusted $p < 0.05$) with 13 (59,1%) GO terms, of which six were identified in the cellular components (CC) category, four in the molecular functions (MF) category and three in the biological processes (BP) category. Additionally, the functional characterization revealed significant enrichment in multiple pathway databases: four (18,2%) pathways in the Human Protein Atlas database, two (9,1%) in the KEGG database, and one (4,5%) each in the WP, REAC and Human Phenotype Ontology databases (Figure 13).

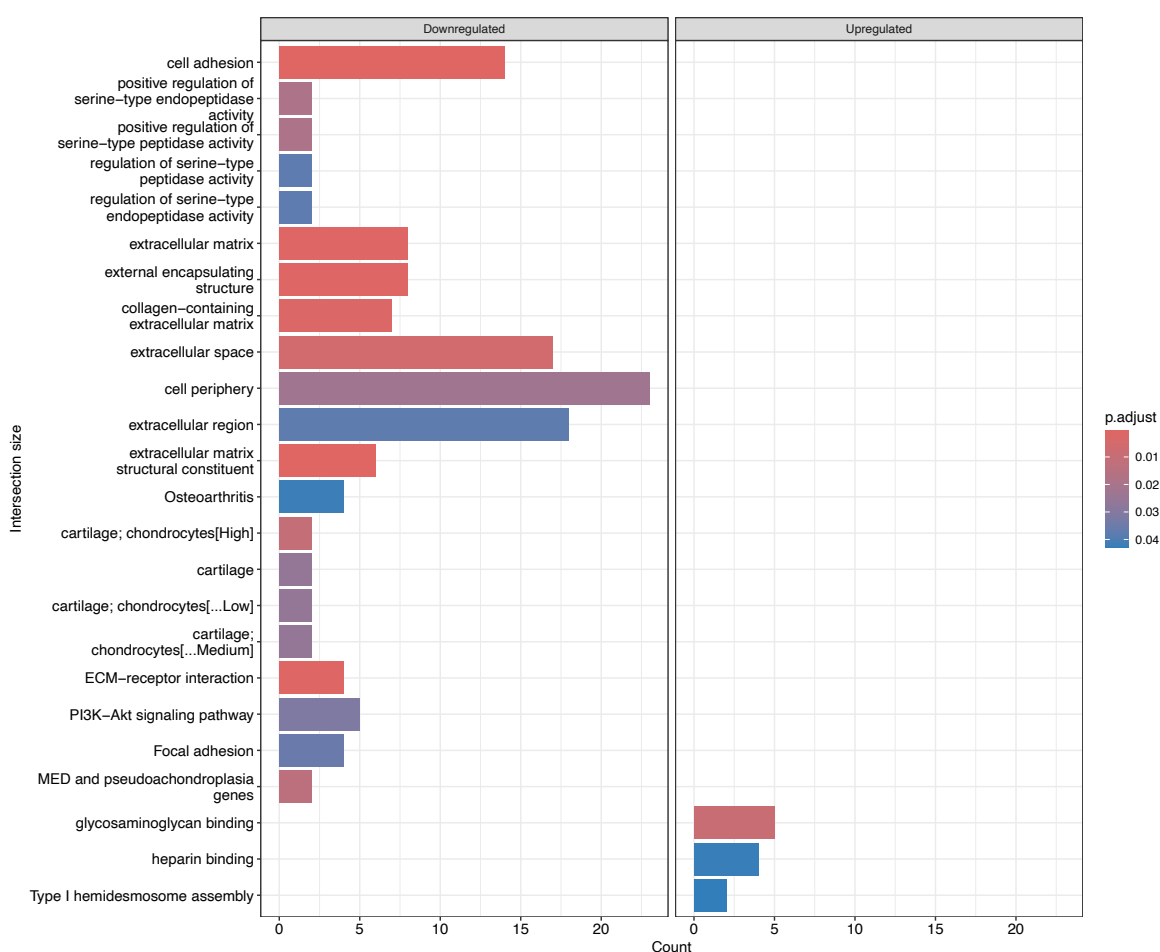


Figure 13: Pathway enrichment analysis of deregulated genes in *PRUNE2* variant carriers: Bar plot displaying the pathways with the highest number of deregulated genes and their statistical significance.

Among the enriched GO terms, those related to the ECM were particularly prominent, including terms associated with cell adhesion, extracellular space, and cell periphery. This emphasis on ECM-related functions was further corroborated by the pathway analysis results from other biological databases. For instance, KEGG analysis

identified pathways significantly impacted by DEGs that are closely related to ECM remodeling, such as ECM-receptor interaction and focal adhesion.

Interestingly, of the 86 DEGs identified in our study, only 37 genes were represented in the significantly enriched pathways (Table 6). Moreover, the majority of these were downregulated, with only six genes being upregulated, suggesting a predominant downregulation of ECM-related genes in *PRUNE2* variant carriers, which could have significant implications for tissue structure and function (Vallet et al., 2022).

Table 6: Deregulated gene associated to enriched pathways in *PRUNE2* variant carriers.

Cluster	Category	Description	p.adjust	geneID	GeneRatio
Downregulated	GO:BP	cell adhesion	0.00065	IGFBP7, THBS4, PCDHA12, PCDHGA3, COL28A1, RELN, SVEP1, ENG, RET, PPFA2, ACAN, COMP, SOX12, PTPRT	14/40
Downregulated	GO:BP	positive regulation of serine-type endopeptidase activity	0.01887	SEMG1, SEMG2	2/40
Downregulated	GO:BP	positive regulation of serine-type peptidase activity	0.01887	SEMG1, SEMG2	2/40
Downregulated	GO:BP	regulation of serine-type peptidase activity	0.03770	SEMG1, SEMG2	2/40
Downregulated	GO:BP	regulation of serine-type endopeptidase activity	0.03770	SEMG1, SEMG2	2/40
Downregulated	GO:CC	extracellular matrix	0.00134	PRG4, IGFBP7, THBS4, COL28A1, RELN, ACAN, COMP, COL9A3	8/40
Downregulated	GO:CC	external encapsulating structure	0.00136	PRG4, IGFBP7, THBS4, COL28A1, RELN, ACAN, COMP, COL9A3	8/40
Downregulated	GO:CC	collagen-containing extracellular matrix	0.00234	PRG4, IGFBP7, THBS4, COL28A1, ACAN, COMP, COL9A3	7/40
Downregulated	GO:CC	extracellular space	0.00586	PRG4, CPZ, IGFBP7, SNCA, THBS4, COL28A1, RELN, SVEP1, ENG, PPFA2, ACAN, CCL14, PGLS, COMP, SEMG1, SEMG2, COL9A3	17/40
Downregulated	GO:CC	cell periphery	0.02260	CSMD2, PTGER3, PRG4, IGFBP7, SNCA, ADH1B, THBS4, PCDHA12, PCDHGA3, COL28A1, RASA4, RELN, OR4F21, ENG, RET, PPFA2, NALCN, ACAN, RGS11, COMP, PTPRT, COL9A3, PDZD4	23/40
Downregulated	GO:CC	extracellular region	0.03762	PRG4, CPZ, IGFBP7, SNCA, THBS4, COL28A1, RELN, SVEP1, ENG, PPFA2, ACAN, CCL14, NXPH3, PGLS, COMP, SEMG1, SEMG2, COL9A3	18/40
Downregulated	GO:MF	extracellular matrix structural constituent	0.00057	PRG4, IGFBP7, COL28A1, ACAN, COMP, COL9A3	6/43
Downregulated	HP	Osteoarthritis	0.04226	PRG4, ACAN, COMP, COL9A3	4/15
Downregulated	HPA	cartilage; chondrocytes[High]	0.01095	ACAN, COL9A3	2/23
Downregulated	HPA	cartilage	0.02618	ACAN, COL9A3	2/23
Downregulated	HPA	cartilage; chondrocytes[Low]	0.02618	ACAN, COL9A3	2/23
Downregulated	HPA	cartilage; chondrocytes[Medium]	0.02618	ACAN, COL9A3	2/23
Downregulated	KEGG	ECM-receptor interaction	0.00149	THBS4, RELN, COMP, COL9A3	4/17
Downregulated	KEGG	PI3K-Akt signaling pathway	0.03154	THBS4, RELN, RET, COMP, COL9A3	5/17
Downregulated	KEGG	Focal adhesion	0.03562	THBS4, RELN, COMP, COL9A3	4/17
Downregulated	WP	MED and pseudoachondroplasia genes	0.01373	COMP, COL9A3	2/23
Upregulated	GO:MF	glycosaminoglycan binding	0.00933	LAMC2, CXCL13, SPOCK3, NELL2, LIPG	5/29
Upregulated	GO:MF	heparin binding	0.04249	LAMC2, CXCL13, NELL2, LIPG	4/29
Upregulated	REAC	Type I hemidesmosome assembly	0.04301	LAMC2, KRT14	2/18

2. Characterization of *PRUNE2* and *PCA3* in prostate cells

To investigate the role of the *PRUNE2*/*PCA3* axis in prostate cells, we started by constructing an siRNA-mediated knockdown model for each individual gene. The model's depletion was validated using a combination of WB, IF, and qRT-PCR assays. These different techniques were performed to, directly and indirectly, determine the optimal time points and concentrations for the effective depletion of *PRUNE2* and *PCA3*.

2.1. Optimization of the *PRUNE2* antibody

The *PRUNE2* antibody was tested in two prostate-derived cell lines, specifically in the non-tumorigenic PNT1A, and the tumorigenic LNCaP cell lines (Figure 14), using three different quantities of protein, 10 μ g, 20 μ g, 30 μ g, and 60 μ g.

The number of bands detected by the WBs differed between the two cell lines. In PNT1A cells, bands were detected at ~100 kDa and ~250 kDa (Figure 14A), while LNCaP cells exhibited several other bands with different molecular weights (Figure 14B). For PNT1A, the detection limit was 20 μ g, with an optimal detection range being determined between 30 and 60 μ g. In the case of LNCaP cells, *PRUNE2* was detectable at a lower protein concentrations, with the optimal amount being between 20 and 30 μ g.

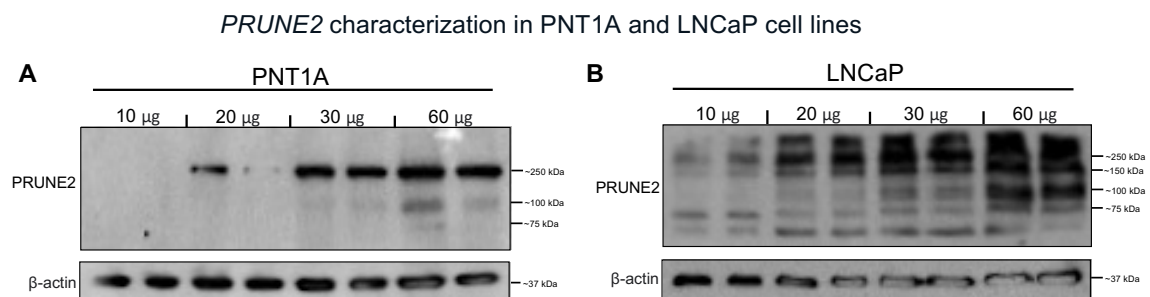


Figure 14: Characterization of *PRUNE2* antibody in PNT1A and LNCaP cell lines. Western blot analysis was performed using 10 μ g, 20 μ g, 30 μ g, and 60 μ g of protein tested against [A] PNT1A and [B] LNCaP cell lines. The *PRUNE2* Rb polyclonal antibody was used to detect *PRUNE2*, and β -actin was used as a loading control.

2.2. Evaluation of siRNA-mediated *PRUNE2* knockdown model

To establish the *PRUNE2* knockdown models, we tested two concentrations of *PRUNE2* siRNA and two time points of siRNA exposure for both PNT1A and LNCaP cell lines. The knockdown efficiency was directly evaluated by WB and IF.

In LNCaP cells, most of the bands detected with the PRUNE2 antibody were depleted with both siRNA concentrations tested (25 nM and 50 nM), more evident using the higher concentration (Figure 15A). Quantification of these bands revealed that at 96h, PRUNE2 levels were reduced by 40-85%, depending on the band being quantified. For PNT1A cells, on the other hand, the depletion of PRUNE2 was not as uniform across bands, with some bands persisting to appear in the WB at the 72h incubation time point (Figure 15B). In PNT1A cells, quantification of these bands revealed that at 96h, PRUNE2 levels were reduced by 50-70%, with the exception of band 4 (~25kDa) which was unaffected by the siRNA treatment. Nonetheless, most of the bands were successfully depleted at 96h. Based on the obtained results, we determined that the optimal conditions for depletion of PRUNE2 are to use 50 nM of siRNA for 96h, for both cell lines.

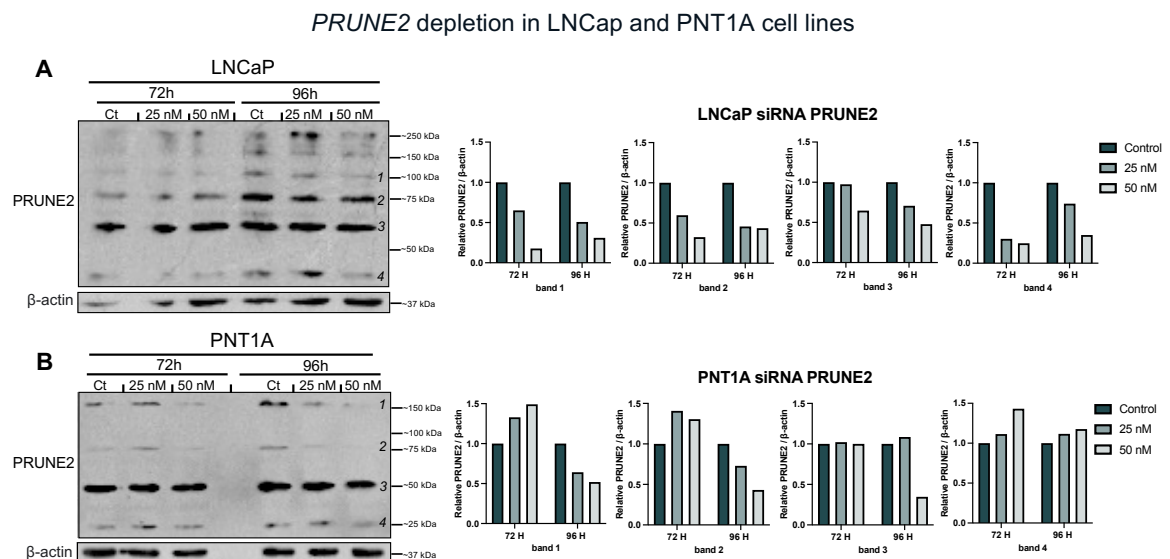


Figure 15: Monitoring the efficiency of PRUNE2 depletion in LNCaP and PNT1A cell lines by WB. Representative protein blot probed to reveal PRUNE2 for [A] LNCaP and [B] PNT1A cells. β -actin was used as a loading control. Quantifications of the four most prominent bands detected are shown on the right for each protein blot. Assays were performed with 25 nM and 50 nM of *PRUNE2* siRNA, for 72h and 96h.

To further validate the siRNA-mediated PRUNE2 depletion, IF assays were performed at 96h with 50 nM siRNA. Additionally, IF analysis was also used to determine the subcellular localization of PRUNE2. To this end, we quantified PRUNE2 levels in both PNT1A and LNCaP cells.

Interestingly, PRUNE2 exhibited different subcellular localization patterns between in PNT1A and LNCaP cells (Figure 16). In control PNT1A cells, PRUNE2 was detected at the cytoplasm and nucleoli. However, upon PRUNE2 depletion, PRUNE2 protein levels

were unchanged in nucleoli, but significantly reduced in the cytoplasm by ~70% (Figure 16 A, B), consistent with the results previously obtained by WB. These data suggest that the nucleoli-associated fraction is insensitive to the siRNA-mediated protein depletion. Conversely, in LNCaP cells, *PRUNE2* was observed to predominantly localize to the cytoplasm, with limited presence in the nucleus. Consistently, *PRUNE2* depletion in these cells resulted in a marked reduction of the protein in the cytoplasm by ~60% (Figure 16 C, D).

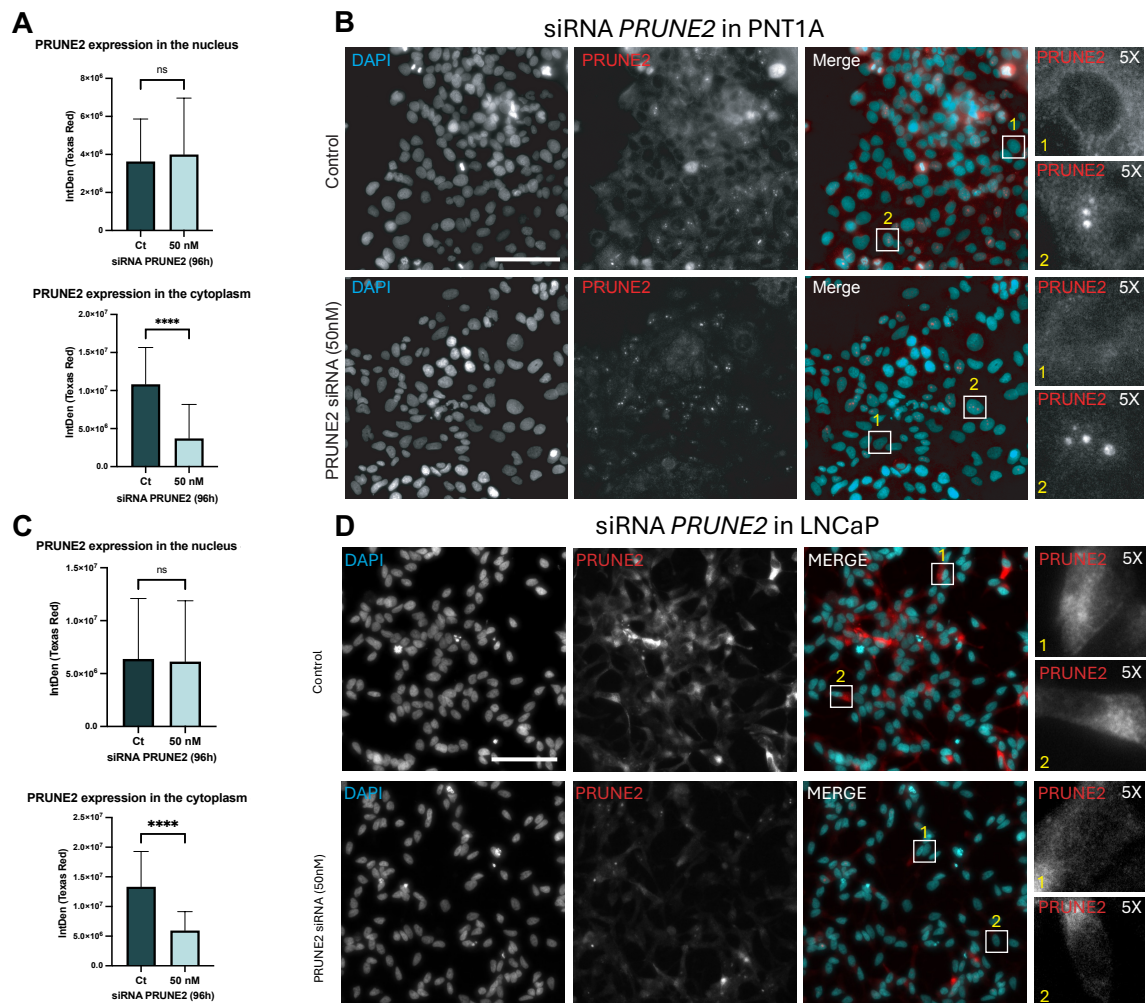


Figure 16: Evaluation of *PRUNE2* levels and subcellular localization in PNT1A and LNCaP cells, using IF assays. [A] Quantifications of the intensity of *PRUNE2* per cell, distinguished between nuclear and cytoplasmatic fractions for PNT1A and LNCaP cells. Statistical analysis was performed using the unpaired two-tailed t-test (**** $p < 0.0001$; ns – not statistically significant). [B] Representative immunofluorescence images comparing Control and *PRUNE2* siRNA-treated cells, in both PNT1A and LNCaP cell lines. Nuclei were stained with DAPI (blue), and *PRUNE2* was stained with the *PRUNE2* Rb polyclonal antibody (red). Enlarged images on the right (5x amplification) highlight the different sub cellular localizations of *PRUNE2* in both cell lines. Images were acquired using the Cytation C10 confocal microscope with a 40x magnification. Representative images were enhanced for better visualization. Scale bar: 100 μ m.

2.3. Evaluation of siRNA-mediated *PCA3* knockdown model

Similarly to PRUNE2, a siRNA-mediated *PCA3* knockdown model was also established in PNT1A and LNCaP cell lines, using two different time points. The depletion efficiency of this target was initially indirectly evaluated by WB and IF, through assessment of PRUNE2 levels. Despite being a long non-coding RNA, the expression pattern of *PCA3* correlates inversely with that of PRUNE2, allowing us to indirectly evaluate its depletion by WB, using the PRUNE2 antibody. *PCA3* depletion was also subsequently validated by quantitative RT-PCR.

To determine *PCA3* siRNA-mediated depletion, we performed siRNA experiments using 25 nM or 50 nM of siRNA for 48h or 72h (Figure 17). Similarly to what we observed in previous experiments, we detected several bands in LNCaP control cells. However, upon *PCA3* depletion, we observed a significant increase in PRUNE2 levels in bands 1-2 ranging from 2 to 3-fold in LNCaP cells (Figure 17A), at the 72h time-point. These results suggest that *PCA3* depletion is likely most efficient at 72h, using 25-50 nM of *PCA3* siRNA.

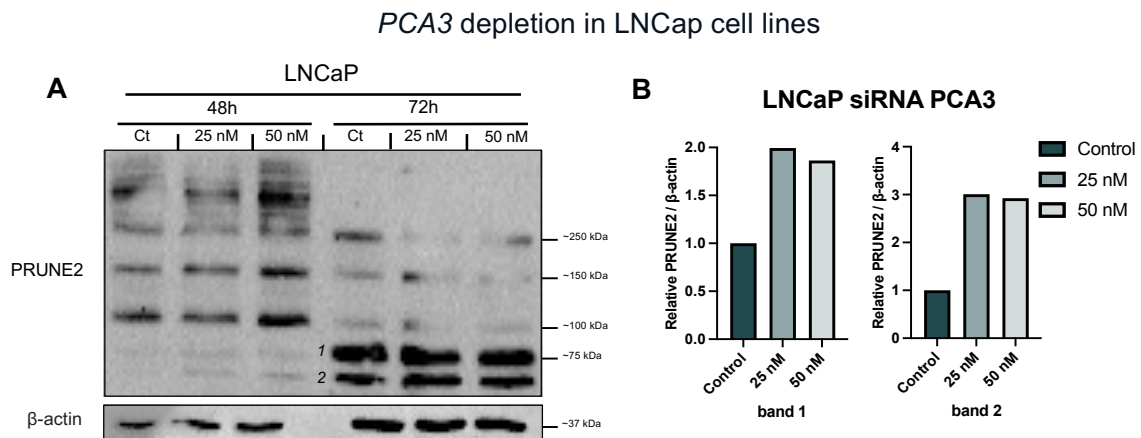


Figure 17: Indirect evaluation of *PCA3* depletion efficiency in LNCaP cells by WB. Representative protein blot probed to reveal PRUNE2 for LNCaP cells. β -actin was used as a loading control. Quantifications of bands 1 and 2, detected at 72h, are shown on the right. Assays were performed with 25 nM and 50 nM of *PCA3* siRNA, for 48h and 72h.

In addition to WB, IF was employed to assess the impact of *PCA3* depletion on PRUNE2 levels and its subcellular localization. IF assays were performed in both PNT1A

and LNCaP cell lines, after transfection with 50 nM of *PCA3* siRNA, for two time points (72h and 96h).

In *PCA3*-depleted LNCaP cells, we observed a significant increase in both cytoplasmic and nuclear PRUNE2 levels. This effect was more evident at the 72h time point, where PRUNE2 levels were increased by ~2-fold in the cytoplasm and in nuclei (Figure 18 A, B), as opposed to the 96h time point (Figure 18 C, D).

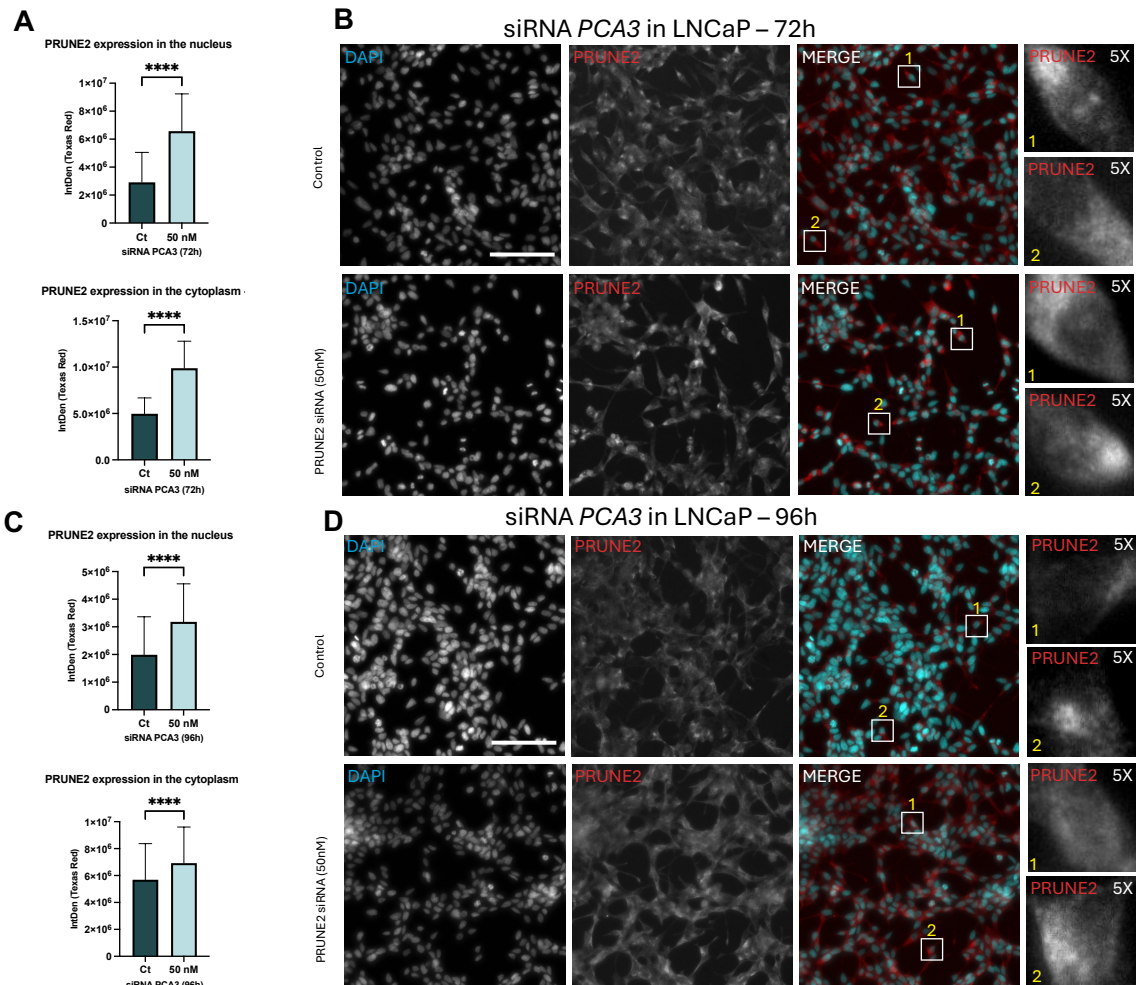


Figure 18: Indirect evaluation of *PCA3* depletion levels in LNCaP cells. [A, C] Quantifications of PRUNE2 intensity per cell, distinguishing between nuclear and cytoplasmatic staining in LNCaP cells, at the 72h and 96h time points, respectively. Statistical analysis was conducted using an unpaired two-tailed t-test (**** $p < 0.0001$; ns – not statistically significant). [B, D] Immunofluorescence images showing LNCaP Control and *PCA3* siRNA-treated cells, for 72h and 96h, respectively. Nuclei are stained with DAPI (blue), and PRUNE2 is stained with the *PRUNE2* Rb polyclonal antibody (red). Higher magnification insets on the right (5x amplification) highlight the different localizations of PRUNE2 for both time points. Images were obtained using the Cytation C10 confocal microscope with a 40x magnification. Representative images were contrasted for better visualization. Scale bar: 100 μ m.

In PNT1A cells, *PCA3* depletion at 72h resulted in an increase of ~2-fold and ~3-fold for nuclear and cytoplasmic fractions, respectively (Figure 19 A, B). The effects of *PCA3* depletion failed to be detected at 96h, where we observed no significant changes in the nuclear and cytoplasmic PRUNE2 levels (Figure 19 C, D). Taken together, these results suggest that *PCA3* depletion is likely most effective at 72h in PNT1A cells.

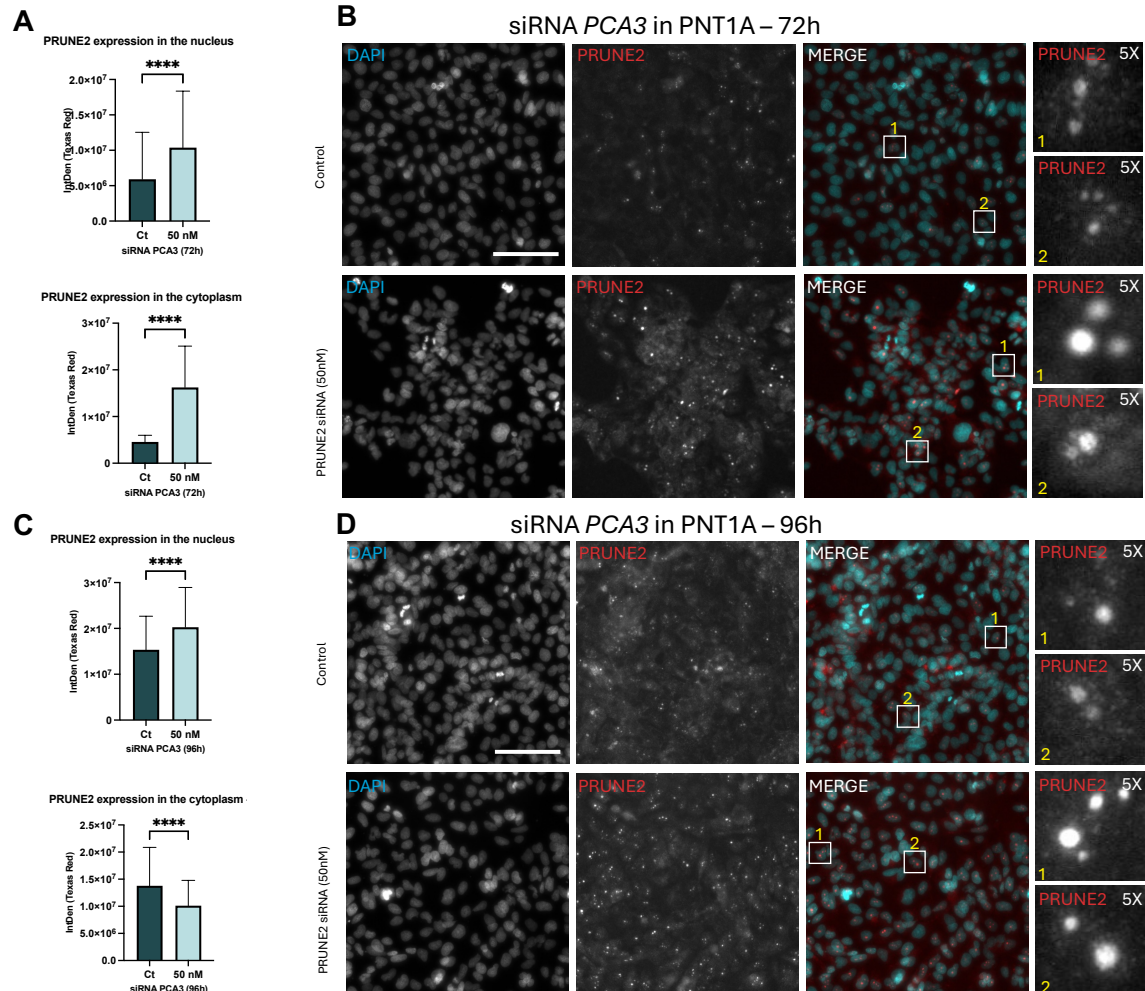


Figure 19: Indirect evaluation of *PCA3* depletion levels in PNT1A cells. [A, C] Quantification of nuclear and cytoplasmic PRUNE2 expression levels per cell, in PNT1A cells at 72h and 96h, respectively, after the addition of *PCA3* siRNA. Statistical analysis was performed using an unpaired two-tailed t-test (**** $p < 0.0001$; ns – not statistically significant). [B, D] Immunofluorescence images showing PNT1A Control and *PCA3* siRNA-treated cells at 72h and 96h, respectively. Nuclei are stained with DAPI (blue), and PRUNE2 was detected with the PRUNE2 Rb polyclonal antibody (red). Higher magnification insets on the right (5x), highlight the localization of PRUNE2 at nucleoli at 72h and 96h. Images were obtained using the Cytation C10 confocal microscope with a 40x magnification. Representative images were contrasted for better visualization. Scale bar: 100 μ m.

Taken together, these results suggest that the optimal conditions for *PCA3* depletion involve treating cells with 50 nM of *PCA3* siRNA for 72h. However, since the quantification of *PRUNE2* levels represents an indirect assessment of *PCA3* knockdown efficiency, we determined the relative levels of *PCA3* using qRT-PCR for both LNCaP and PNT1A cell lines (Figure 20). In agreement with our previous results, we observed that *PCA3* mRNA levels were decreased by ~30% in both PNT1A and LNCaP cell lines at 72h. This decrease was maintained in LNCaP cells, where we observed that *PCA3* mRNA expression levels remain diminished for at least 144h. In contrast, we observed that *PCA3* mRNA expression in PNT1A cells was indistinguishable from controls at 144h, consistent with a more transient depletion in this cell line. Taken together with previous results on the indirect assessment of *PCA3* depletion efficiency, we defined treatment with 50 nM of *PCA3* siRNA for 72h, as the optimal conditions for *PCA3* depletion in both PNT1A and LNCaP cells.

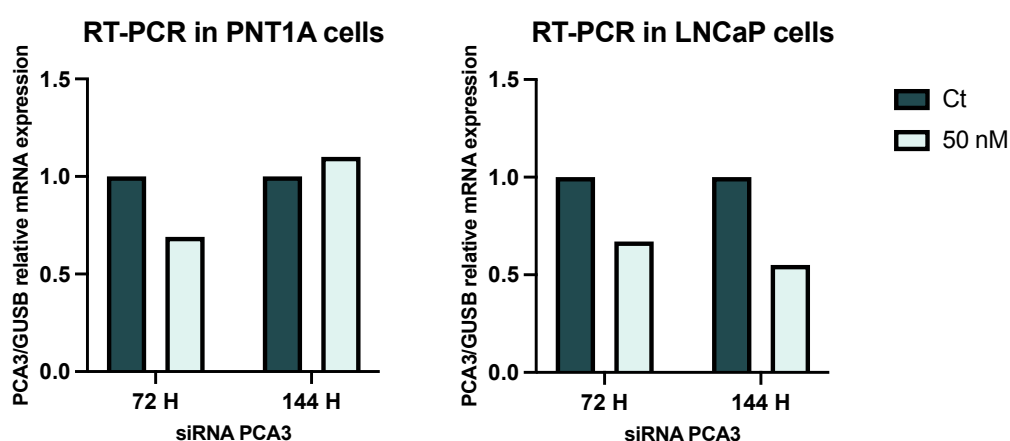


Figure 20: Quantification of *PCA3* mRNA expression levels using qRT-PCR. Relative quantification of *PCA3* mRNA levels following treatment with 50 nM of *PCA3* siRNA for 72h and 144h, in both PNT1A and LNCaP cells. Gene expression was normalized to the housekeeping gene *GUSB*, and the relative quantification calculated using the $\Delta\Delta C_t$ method. Data represents results obtained from one assay, with no independent experiments performed.

3. Effects of androgen therapy in prostate cell lines

To assess the effects of androgen therapy in prostate cell lines, we began by testing different androgen therapies, namely enzalutamide, an androgen antagonist, and DHT, an androgen agonist on wildtype androgen-sensitive prostate cell lines, PNT1A and LNCaP. We began by optimizing the experimental conditions, including testing various cell seeding densities in a 96-well plate format (data not shown). After evaluating different seeding

concentrations, we determined that 10,000 cells per well was optimal for conducting subsequent assays. Following optimization of cell seeding, we conducted titration experiments using a range of enzalutamide and DHT concentrations to determine the most effective doses for assessing their impact on cell viability and death. We used the Calcein AM and PI staining assay to measure the percentage of live and dead cells, respectively, 72h after incubation with varying doses of Enzalutamide and DHT (data not shown). Given the lack of observable effects within the initial 3-day timeframe, we extended the incubation period to 12 days to allow for potential delayed responses to the androgen therapies. Despite the prolonged exposure, no significant variations in cell viability or death were observed within each cell line, even with increasing doses of enzalutamide or DHT. This pattern was consistent across both PNT1A and LNCaP cell lines (Figure 21A). Notably, we did observe a higher percentage of cell death in PNT1A cells (Figure 21B). However, this effect on the percentage of dead cells was independent of the drugs used, since these differences were also clearly observed in untreated controls, which were not exposed to Enzalutamide or DHT.

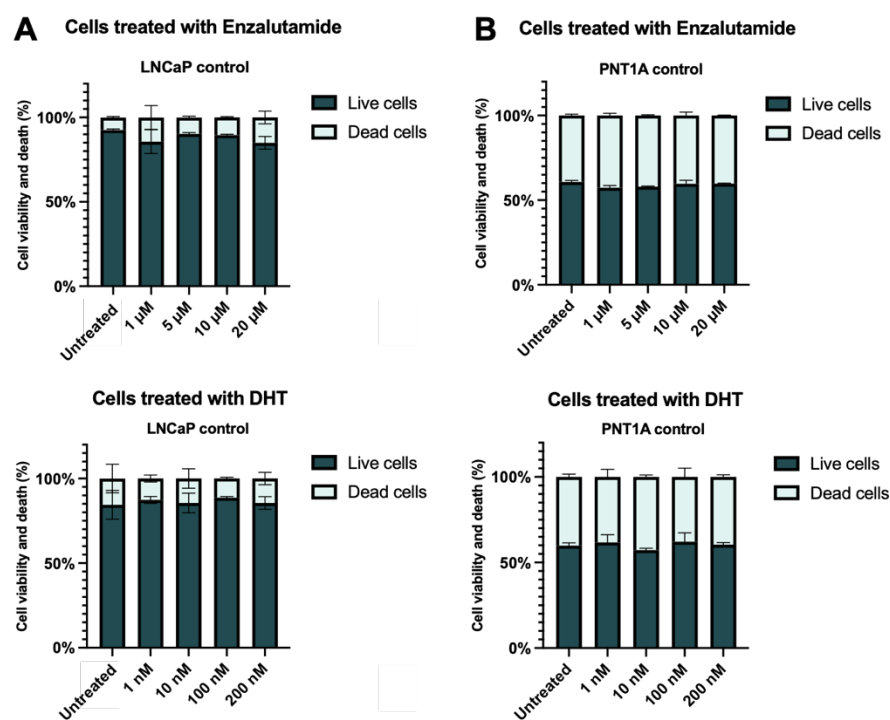


Figure 21: Administration of androgen therapy in wildtype PNT1A and LNCaP cells. Graphs show results for [A] PNT1A, and [B] LNCaP cell lines treated with DMSO, 1 μ M, 5 μ M, 10 μ M or 20 μ M Enzalutamide (anti-androgen), or with DMSO, 1 nM, 10 nM, 100 nM or 200 nM DHT (androgen agonist). Cells were incubated with the treatment for 12 days and cell viability and cell death were measured using the Calcein and PI staining. Each condition was replicated in three wells per treatment, in the same 96-well plate, and bar graphs represent the average percentages of triplicate wells. Error bars represent the standard deviation, n=10 000 cells analysed.

4. Characterization of candidate DEGs and oncogenic signaling pathways

4.1. Characterization of candidate DEGs

Following whole-transcriptome analysis highlighting several DEGs and signaling pathways deregulated in *PRUNE2* variant carriers, we selected promising deregulated genes for further analysis. Among these, *Laminin subunit gamma 2 (LAMC2)*, emerged as a particularly significant candidate due to its role as a precursor of the *PI3K/Akt* signaling pathway, a critical oncogenic pathway frequently deregulated in various cancers. Notably, *LAMC2* was found to be upregulated in *PRUNE2* variant carriers, suggesting a potential link between *PRUNE2* deregulation and the activation of *PI3K/Akt* signaling. Moreover, *LAMC2* demonstrated a significant log₂ fold change of 1.634 ($p = 0.0224$) between *PRUNE2* variant carriers and non-carriers, indicating a substantial upregulation in carriers. Moreover, the expression of *LAMC2* was consistently higher in tumor tissues compared to normal tissues (Figure 22A), regardless of *PRUNE2* variant status (Figure 22B). To further explore the impact of *PRUNE2* variants on the *PI3K/Akt* pathway, we extended our analysis to include two additional key genes, namely *Akt* and B-cell lymphoma 2 (*Bcl-2*), both of which play crucial roles in this pathway. *Akt*, a well-characterized oncogene, displayed higher expression levels in tumor tissues compared to normal tissues, consistent with its established role in promoting oncogenic signaling. However, when comparing its expression levels in carriers and non-carriers, *Akt* expression is higher in non-carriers, particularly in tumor tissues. Lastly, *Bcl-2*, an anti-apoptotic gene crucial for regulating cell survival, was selected due to its role as a downstream component of the *Akt* signaling pathway. Interestingly, *Bcl-2* exhibited higher expression in normal tissues compared to tumor tissues, but was upregulated in *PRUNE2* variant carriers. These data suggest that despite its generally lower expression in tumors, the presence of *PRUNE2* variants may enhance *Bcl-2*-mediated cell survival signals.

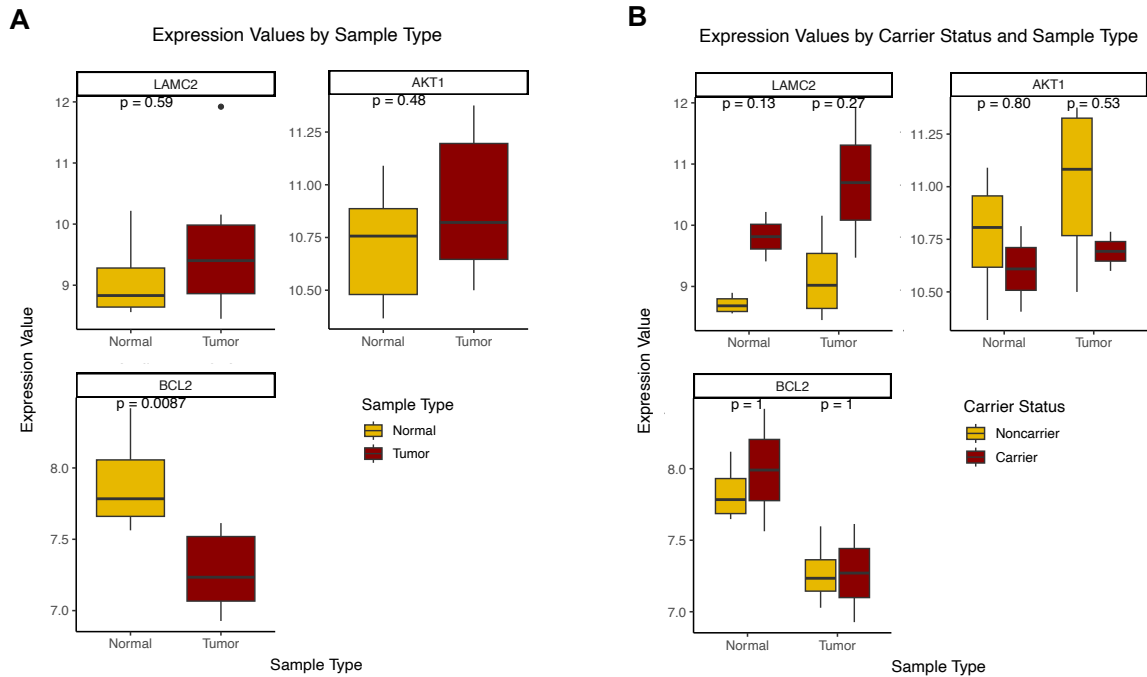


Figure 22: Characterization of promising deregulated genes identified in *PRUNE2* variant carriers. Boxplots reveal the different expression levels (log₂ normalized counts) for *LAMC2*, *Akt* and *Bcl-2* in [A] different sample types (normal and tumor) and in [B] different carrier status (carriers and non-carriers), adjusted for the sample type. $p < 0.05$. $n = 6$.

4.2. Cellular characterization of selected genes in prostate cells

To gain a better understanding of the roles of the promising genes *LAMC2*, *Akt*, and *Bcl-2* in the context of PrCa biology, we conducted a preliminary characterization of the subcellular localization of the proteins they encode in *wildtype* PNT1A and LNCaP cells. IF analysis revealed that *Akt* is predominantly localized to the cytoplasm in both cell lines, although it also a detectable at nuclei, particularly in PNT1A cells (Figure 23). The predominance of *Akt* in the cytoplasm, coupled with its nuclear presence, may indicate its multifaceted role in oncogenic signaling. *LAMC2* was predominantly observed in the cytoplasm and nuclei in LNCaP cells, and at the interface between the nucleus and the cytoplasm (nuclear lamina) in PNT1A cells. This localization may be important in the context of cellular adhesion and communication, since *LAMC2* has been shown to be a component of the ECM. Conversely, *Bcl-2* exhibited a strong nuclear concentration in both cell lines, consistent with its established function as an anti-apoptotic protein. Interestingly, in LNCaP cells, we also observed significant cytoplasmic accumulation of *Bcl-2*. Of particular note, we also observed that *Bcl-2* levels were more heterogeneous within each cell line (particularly

in PNT1A cells), likely reflecting different cell cycle stages and/or apoptotic program regulation.

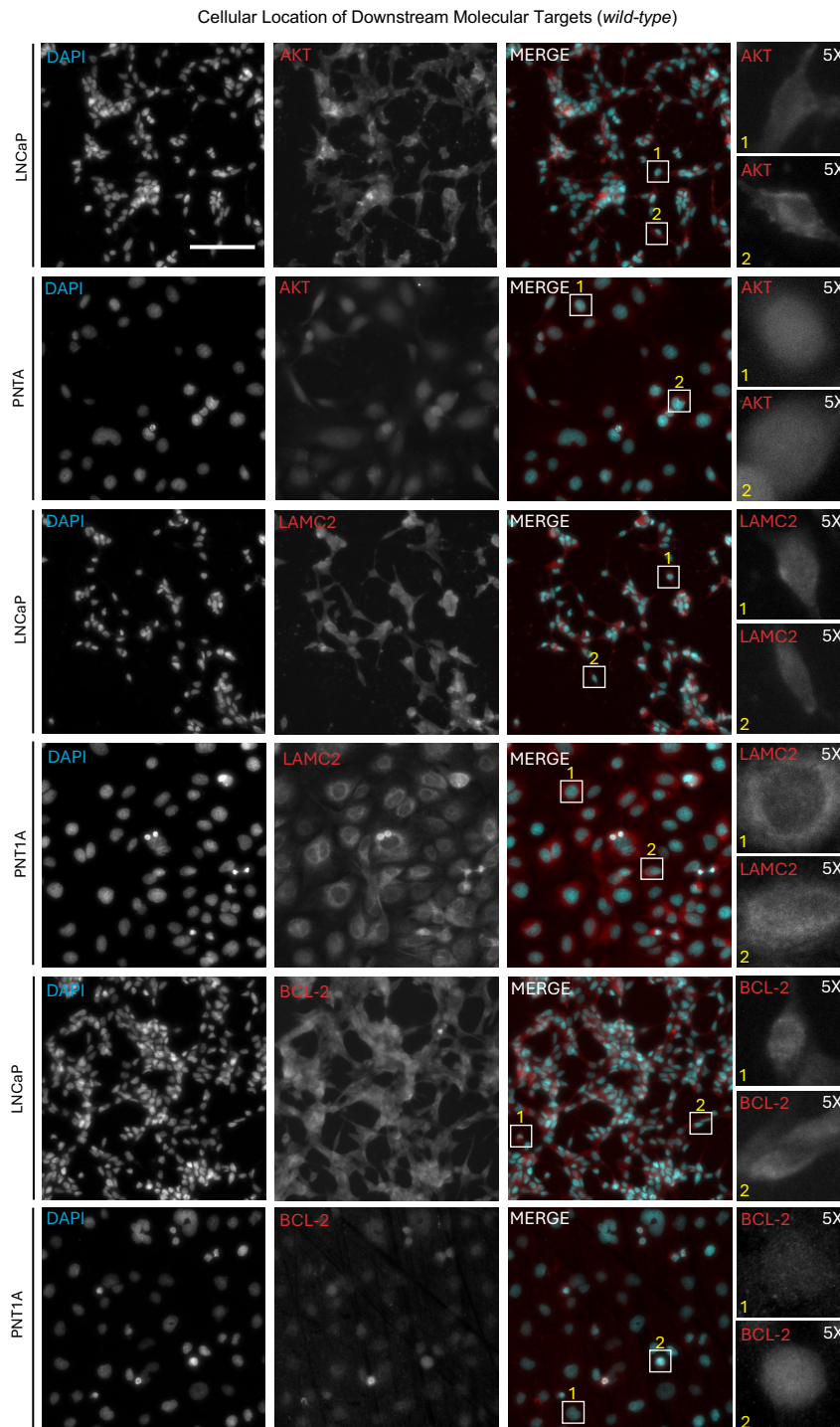


Figure 23: Characterization of promising deregulated genes identified in *PRUNE2* variant carriers. Representative immunofluorescence images showing the different cellular locations of LAMC2, Akt and *i* in both PNT1A and LNCaP cells. Nuclei are stained using DAPI (blue) and the proteins corresponding to the potentially deregulated genes were detected with Akt Ms monoclonal antibody, LAMC2 Ms monoclonal antibody, or Bcl-2 Ms monoclonal antibody, respectively (red). Higher magnification insets (5x amplification) are shown on the right, highlighting representative examples for each immunofluorescence analysis in both LNCaP and PNT1A cell lines. Images were obtained using the Cytation C10 confocal microscope with a 40x magnification. Representative images were contrasted for better visualization. Scale bar: 100 μ m.

DISCUSSION

As previously described, PrCa global burden is quite substantial, representing one of the major causes of oncological disease and mortality among men worldwide (Ng, 2021). Despite family history of the disease being one of the most important risk factors for PrCa development, the genetic mechanisms behind such increased susceptibility are still mostly elusive. A previous study from our group reported pathogenic or likely pathogenic germline variants in the *PRUNE2* gene in 2.8% of 462 HPC patients, making it the most frequently mutated gene in this series (Cardoso et al., 2023). These findings led to the proposal of *PRUNE2* as a PrCa tumor suppressor gene, regulated by the non-coding RNA *PCA3*, the most specific PrCa biomarker described to date (Salameh et al., 2015). *PRUNE2* deregulation, described in several tumor types, including prostate, has been shown to modulate signaling networks and affect multiple cellular functions, including apoptosis (Li et al., 2022; Machida et al., 2006; Zhao et al., 2013).

Building on these previous findings, the primary objective of this study was to investigate the involvement of *PRUNE2* in oncogenic signaling pathways and to further explore the role of the *PRUNE2/PCA3* regulatory axis in PrCa development and progression. We started by analyzing whole-transcriptome data from *PRUNE2* variant carriers and non-carriers, aiming to identify DEGs and deregulated pathways associated with *PRUNE2* alterations. Additionally, we developed knockdown models for both *PRUNE2* and *PCA3* in two androgen-sensitive prostate cell lines: PNT1A, a non-tumorigenic epithelial cell line, and LNCaP, a tumorigenic cell line derived from metastatic PrCa, to study the effects of their depletion *in vitro*, and to better understand the interplay between *PRUNE2*, *PCA3*, and *AR*.

Given the limited existing knowledge regarding the roles of *PRUNE2* and *PCA3* in PrCa, particularly in their involvement in oncogenic signaling pathways, our study sought to provide new insights into the molecular mechanisms driving PrCa development and progression.

1. *PRUNE2* variant carriers exhibit a distinct transcriptomic profile with several deregulated pathways

Whole-transcriptome analysis of the two *PRUNE2* variant carriers and four non-carriers allowed us to investigate the expression patterns of *PRUNE2*, and its interacting partner *PCA3*, as well as the implications of *PRUNE2* alterations on the transcriptomic

landscape of its carriers. Our findings indicate that *PRUNE2* expression is lower in tumor tissues, while *PCA3* is increased in the same tissues. Furthermore, in *PRUNE2* variant carriers, we observed a reduced expression of this gene, suggesting a potential reduction in function for patients harboring this genetic alteration. In line with this, previous work by Salameh *et al.* established a regulatory axis between *PRUNE2* and *PCA3* in PrCa, where *PCA3* was shown to downregulate *PRUNE2*, while the silencing of *PCA3* or overexpressing *PRUNE2* reverses this effect (Salameh et al., 2015). Although our results did not reach statistical significance, likely due to the small sample size, the observed trends – lower *PRUNE2* and higher *PCA3* expression levels in tumors – are consistent with prior studies (Lauer et al., 2023).

86 deregulated genes in *PRUNE2* variant carriers compared to non-carriers, of this 46 were downregulated and 40 were upregulated. These findings suggest that variations in *PRUNE2* could affect several cellular processes. To investigate the potential biological impact of these DEGs, we performed pathway enrichment analysis. Of the 86 deregulated genes, 37 were linked to multiple well-known cellular processes commonly altered in cancer. Specifically, many of the enriched pathways were related to cell adhesion, the ECM, or the extracellular space. The ECM plays a pivotal role in providing tissue strength and support, being composed of several macromolecules such as collagen, fibronectins, and laminins (Dzobo & Dandara, 2023). Several of the deregulated genes identified, such as Laminin subunit $\gamma 2$ (*LAMC2*), Insulin-like growth factor binding protein-7 (*IGFBP7*), and Thrombospondin 4 (*THBS4*), are associated with ECM remodeling, which plays a crucial role in cancer progression by influencing various signaling pathways, including the *Akt* pathway (Akiel et al., 2017; Erice et al., 2023; Guo et al., 2020).

LAMC2, upregulated in *PRUNE2* variant carriers, encodes the protein Laminin $\gamma 2$, a subunit of the glycoprotein Laminin-332, essential in regulating cell adhesion and mobility (Cave et al., 2022). This gene has also been linked to the regulation of the epithelial-mesenchymal transition (EMT) and is associated with a worse prognosis in pancreatic cancer (Cave et al., 2022). Erice *et al.* recently linked *LAMC2* to the upregulation of *Akt* via phosphorylation, thereby promoting cell survival and invasion via the *FOSL1*-*AXL* axis (Erice et al., 2023). Similarly, *IGFBP7* has also been implicated in several types of cancer, including PrCa, hepatocellular, breast, colon, and lung carcinomas, by regulating cell proliferation and apoptosis (Sullivan et al., 2012). *IGFBP7*, downregulated in *PRUNE2* variant carriers, acts as a tumor suppressor by blocking the IGF-1 receptor to inhibit IGFs activation further. Moreover, Akiel and colleagues observed activation of *Akt* in mice lacking *IGFBP7* expression, due to the activation of

the IGF-1 receptor, suggesting that *IGFBP7* is essential for downregulating *Akt* and promoting apoptosis (Akiel et al., 2017). Lastly, *THBS4*, also downregulated in *PRUNE2* variant carriers, is responsible for cell-to-cell adhesion, cytoskeleton formation, and embryonic development (Guo et al., 2020). This gene acts as a tumor suppressor in colorectal cancer (Greco et al., 2010), and as an oncogene in hepatocellular carcinoma, by promoting the expression of *N-cadherin* (important for the EMT), and by facilitating the phosphorylation of *PI3K* and *Akt* (Guo et al., 2020). *Akt* is among the most important altered oncogenic targets, playing a critical role in cancer progression and survival (Revathidevi & Munirajan, 2019).

Deregulation of the aforementioned genes in *PRUNE2* variant carriers suggests that alterations in *PRUNE2* might contribute to the activation of *Akt*, potentially promoting cell survival. This is consistent with the work of Tatsumi *et al.* in neuroblastomas, where they demonstrated that *PRUNE2* inhibits *Akt* phosphorylation, thereby preventing the activation of the pathway (Tatsumi et al., 2015). Interestingly, *Akt* gene expression was increased in tumor tissues but decreased in *PRUNE2* carriers compared to non-carriers. However, since the RNA-sequencing analysis did not account for phosphorylated *Akt*, it is possible that *PRUNE2* variant carriers have higher levels of phosphorylated *Akt*, indicating activation of the *Akt* pathway and promotion of cell survival. To further investigate the potential impact of *PRUNE2* alterations on the *Akt* pathway, we also investigated the expression of the oncogene *Bcl-2*, a known downstream target of the *Akt* pathway. Our results showed that *Bcl-2* was increased in *PRUNE2* variant carriers compared to non-carriers. This upregulation suggests an increased activation of the *Akt* signaling pathway in *PRUNE2* variant carriers.

To further evaluate the impact of *PRUNE2* deregulation in the *Akt* signaling pathway, we also performed IF assays to evaluate their subcellular localization in the androgen-sensitive prostate cells. Based on their function, *LAMC2* is primarily expected to be localized in the extracellular matrix and at the cell membrane, where it plays a role in cell adhesion and migration (Cheng et al., 2024), whereas *Bcl-2*, as an anti-apoptotic protein, is predominantly found in the nucleus (Hoetelmans et al., 2000). *Akt* has been reported to exhibit a dynamic localization, moving between the cytoplasm and nucleus depending on its activation state (Le Page et al., 2006). IF assays performed to detect *LAMC2*, *Akt* and *Bcl-2* localization in wildtype PNT1A and LNCaP cells were consistent with previous studies. *Akt* was predominantly localized to the cytoplasm in both cell lines, while *Bcl-2* exhibited a strong nuclear concentration in both cell lines. Interestingly, *LAMC2* was predominantly expressed in the cytoplasm and nuclei in LNCaP cells, and at the nuclear lamina, between the nucleus and the cytoplasm, in PNT1A cells. Following

the reported association between the *AR* and the Akt pathway, it is paramount to investigate the localization and abundance of these genes in the context of *PRUNE2* deregulation. Therefore, these findings will be used as baseline for future studies with *PRUNE2*- and *PCA3*-depleted cell lines.

2. Complex landscape of *PRUNE2* isoforms

The detection of *PRUNE2* protein by WB revealed several bands of varying molecular weights in non-tumorigenic PNT1A and tumorigenic LNCaP cells. While some of these bands were common to both cell types, others were unique to the LNCaP cells. All bands were detected using an antibody specific to *PRUNE2*, suggesting that these bands represent distinct isoforms. This is further supported by the significant and consistent ~50% reduction in all detected bands in both PNT1A and LNCaP cells following *PRUNE2* siRNA treatment indicating that the multiple bands likely correspond to different *PRUNE2* isoforms.

PRUNE2 is a complex gene with at least 25 exons, transcribed into 14 transcripts, half of those translated into protein (Martin. et al., 2023). According to the GTEx Portal, the canonical form contains 19 exons, although this is not the most expressed isoform (Lonsdale et al., 2013). The most expressed isoform (ENST00000376717) contains 11 exons and produces a protein of approximately 40 kDa (Martin. et al., 2023). Despite the calculated molecular weight of *PRUNE2* being 340 kDa, the antibody used in our research, as well as in other relevant studies (Li et al., 2022; Salameh et al., 2015; Tatsumi et al., 2015), primarily recognizes a band at ~150 kDa (Group, 2024), corresponding to the first seven exons of the canonical isoform. This limitation prevents the detection of the most expressed isoform, which is produced after exon 15, implying that several isoforms, including the most abundant one, may have been undetected in our assays. Interestingly, Salameh *et al.* also detected a band at ~450 kDa in LNCaP cells using the same antibody (Salameh et al., 2015). Combined with our observation of multiple band depletion after *PRUNE2* siRNA treatment in both LNCaP and PNT1A cells, these findings suggest that we may be detecting more isoforms of *PRUNE2* than initially anticipated. Future studies using alternative *PRUNE2* antibodies may provide deeper insights into the abundance and functional roles of the various *PRUNE2* isoforms, advancing our understanding of *PRUNE2*'s role in HPC.

Given the elevated number and complexity of *PRUNE2* isoforms, employing an alternative depletion model, such as CRISPR-Cas9, could provide more comprehensive insights. CRISPR-Cas9 allows for precise genome editing, enabling the complete

ablation of *PRUNE2* expression and the generation of cell lines entirely lacking the protein (Ma et al., 2014). However, despite the advantages of CRISPR, in our study, siRNA was chosen as it provides a partial depletion of gene expression, thus allowing to investigate the effects of reduced *PRUNE2* and *PCA3* expression, which may resemble the potential partial loss of function observed in patients with heterozygous variants.

3. *PRUNE2* localizes to different subcellular locations in PNT1A and LNCaP cells

IF analysis revealed intriguing findings regarding the subcellular localization of *PRUNE2* in non-tumorigenic (PNT1A) and tumorigenic (LNCaP) cells. In LNCaP cells, *PRUNE2* was primarily localized in the cytoplasm and excluded from the nucleus. In contrast, in PNT1A cells, *PRUNE2* was predominantly concentrated in the nucleoli, although a smaller portion of the protein was still detectable in the cytoplasm.

According to The Human Protein Atlas, *PRUNE2* is predominantly cytoplasmic, as observed in the GAMG (glioblastoma) and Rh30 (rhabdomyosarcoma) cell lines (Uhlén M, 2015). Our findings in LNCaP cells are consistent with those of Salameh *et al.*, who also reported cytoplasmic localization of *PRUNE2* in these cells (Salameh et al., 2015). However, the strikingly different localization of *PRUNE2* in PNT1A cells raises important questions about its distinct roles in tumorigenic versus non-tumorigenic contexts. To date, studies on *PRUNE2* have mainly focused on tumorigenic cells, leaving a gap in knowledge regarding its function and localization in non-tumorigenic environments. One possibility is that *PRUNE2* has a nuclear function in non-tumorigenic cells that is lost or altered upon malignant transformation. Alternatively, *PRUNE2* may shuttle between the cytoplasm and nucleus, with its localization and function being regulated by different mechanisms in normal versus cancer cells. This cytoplasm-nucleus shuttling could be disrupted in cancer, potentially contributing to tumorigenesis.

In fact, several other key genes, frequently altered in cancer, exhibit different subcellular localizations depending on the cellular environment. For example, *TP53*, one of the most studied tumor suppressor genes, encodes the protein p53, which accumulates in the nucleus in response to DNA damage to promote cell cycle arrest and apoptosis (Iwahashi et al., 2022). However, in certain cancers like retinoblastoma, colorectal carcinoma, and breast adenocarcinoma, p53 is sequestered in the cytoplasm, preventing its nuclear translocation and thereby inhibiting its ability to trigger cell death

(O'Brate & Giannakakou, 2003). Similarly, in PrCa, Cai *et al.* reported that p53 was unable to enter the nucleus in LNCaP cells due to the negative regulation by the androgen-regulated gene *sGC α 1* (Cai *et al.*, 2012). *BRCA1*, another well-known tumor suppressor involved in DDR, cell cycle regulation, and apoptosis, is primarily located in the nucleus of non-tumorigenic cells (Takaoka & Miki, 2018). It shuttles between the nucleus and cytoplasm via a nuclear export signal in its N-terminal region (Fabbro *et al.*, 2002). Mutations in *BRCA1*'s C-terminus can cause its mislocalization to the cytoplasm, impairing its tumor-suppressive functions (Drikos *et al.*, 2021). Interestingly, Wiener *et al.* reported that *BRCA1* and its binding partner *BARD1*, which normally retains *BRCA1* in the nucleus, colocalize in the cytoplasm in 51.4% of breast cancer tissues analyzed (n = 103) (Wiener *et al.*, 2015).

The different cellular localizations of *PRUNE2* in PNT1A and LNCaP cells could indicate a mechanism that is altered in tumorigenic cells and may represent a targetable pathway. Conducting future experiments using a nuclear localization signal (NLS) to specifically direct *PRUNE2* to the nucleus in LNCaP cells may provide critical insights into its distinct roles in different cellular compartments. Moreover, it would be essential to test additional cell lines to determine whether these localization differences are exclusive to PNT1A and LNCaP cells or if they are a common feature that distinguishes normal from cancerous cells.

The cellular localization of *PRUNE2* may be influenced by the distinct isoform patterns observed in our IF assays, as different isoforms are likely to vary in both function and cellular localization. These isoforms may exert diverse roles depending on whether they are localized in the nucleus or cytoplasm, potentially added a new layer of regulation which can contribute to different cellular processes in non-tumorigenic and tumorigenic contexts.

4. Monitoring the Effects of Anti-Androgen Therapy

In our experiments, we did not observe any significant differences in cell viability or cell death in either LNCaP or PNT1A cells following treatment with varying amounts of Enzalutamide (an AR inhibitor) or DHT (an endogenous androgen), even after prolonged treatment (12 days) with the highest concentrations of Enzalutamide and DHT. The only notable differences we observed were between the two cell lines themselves, likely reflecting intrinsic differences between PNT1A and LNCaP cells. LNCaP cells exhibited lower levels of cell death, which aligns with the expected decrease in apoptosis in tumorigenic cells. However, caution is necessary when interpreting these

results, as the LNCaP cell line is known for its weaker adherence to culture plates, leading to a higher likelihood of cell detachment during our experiments. This poor adhesion, previously reported as a characteristic of LNCaP cells (Liberio et al., 2014), could have contributed to the observed results. Specifically, the Calcein AM and PI assay used to assess live and dead cells required the removal of culture media followed by a PBS wash, which in some cases resulted in cell loss, particularly for LNCaP cells.

There is also conflicting evidence regarding PNT1A's sensitivity to anti-androgen therapy. According to the literature, PNT1A, a benign prostate cell line, expresses low levels of the *AR* mRNA and no detectable *AR* protein (Coll-Bastus et al., 2015). Based on this, PNT1A cells to be less sensitive to androgen deprivation therapies. Our results reflect a potential insensitivity, even at high doses and extended incubation times. As for LNCaP cells, previous studies have shown varied responses to anti-androgen therapies. For instance, Özgür *et al.* investigated the effects of Enzalutamide on cell viability using a *PCA3* knockdown model, with the greatest impact observed in LNCaP-*AR*⁺ cells (Ozgur et al., 2017). This suggests that the LNCaP cells used in our assays might have developed insensitivity to *AR*-targeting treatments, which could explain why we did not observe the expected outcomes following the administration of Enzalutamide or DHT. Although we monitored cell viability and death, it is possible that *AR* modulation occurred without significantly affecting these endpoints. Measuring alternative markers, such as *PSA* levels, may provide a more sensitive indicator of *AR* activity. Despite this, the lack of response to Enzalutamide and DHT in our prostate cell lines is unusual and warrants further studies. In fact, given these surprising findings and apparent insensitivity to androgen treatments observed in both our LNCaP and PNT1A cells, we have decided to postpone the evaluation of androgen treatment effects in *PRUNE2*- and *PCA3*-depleted cell models. This decision is based on the critical need to first investigate several of the aforementioned hypotheses that could explain the observed lack of response, in order to accurately assess the roles of *PRUNE2* and *PCA3* in *AR* signaling.

CONCLUSION

Overall, this study provides novel insights into the involvement of *PRUNE2* in PrCa, and its interaction with the non-coding RNA *PCA3*. While previous work has suggested that *PRUNE2* acts as a tumor suppressor in PrCa, our findings shed light on its potential involvement in oncogenic pathways and functional diversity. Specifically, we conclude that:

- *PRUNE2* variant carriers exhibited a distinct transcriptomic profile compared to non-carriers with several deregulated genes, many of which are linked to cancer-related processes and pathways, including those involved in ECM remodeling and cell adhesion. Notably, deregulated genes such as *LAMC2*, *IGFBP7*, and *THBS4* were associated with the *PI3K/Akt* pathway activation, highlighting the potential influence of *PRUNE2* on this key oncogenic signaling cascade.
- *PRUNE2* exhibited different isoform patterns depending on the non-tumorigenic (PNT1A) and tumorigenic (LNCaP) contexts, as well as subcellular localizations, suggesting that its function and localization may change during tumorigenesis.
- Our *wildtype* PNT1A and LNCaP cells were largely insensitive to *AR* modulation using Enzalutamide (*AR* antagonist) or DHT (*AR* agonist), indicating possible androgen receptor-targeting therapy insensitivity, or limitations in used cell viability detection methods. These need to be assessed to accurately investigate the roles of *PRUNE2* of *PCA3* in *AR* signaling.

FUTURE PERSPECTIVES

The results of this work have raised several questions that we would like to address in the future. Firstly, multiple *PRUNE2* isoforms were detected in this study, yet their specific roles remain unclear. For a better characterization of the functional differences of these isoforms, several methods could be employed. For instance, we would like to use alternative antibodies that can detect other *PRUNE2* isoforms to obtain a more complete picture of their abundance, localization and role in PrCa. Additionally, long-read sequencing also presents an interesting approach for characterizing different *PRUNE2* isoforms. Unlike short-read sequencing, long-read technologies generate longer reads that can span complex genomic regions, including alternative exons and junction regions, thus producing a more comprehensive view of *PRUNE2* isoforms.

Secondly, following our findings regarding *PRUNE2*'s different subcellular localization in LNCaP and PNT1A cells, we plan to further investigate its localization in additional tumorigenic and non-tumorigenic cell lines.

Thirdly, the lack of response to androgen therapy in this study suggests the need to perform additional experiments either with higher drug concentrations or more prolonged treatments, to evaluate the treatment sensitivity. Therefore, we plan to perform additional assays with different drug titrations and timepoints in *wildtype*, as well as extend all the assays to *PRUNE2*- and *PCA3*-depleted cell models. Furthermore, we will also use new detection methods, such as *PSA* quantification, to evaluate the treatment effect in all experiments.

Lastly, given the deregulation of *Akt* signaling-related genes in *PRUNE2* variant carriers, future research should focus on assessing the impact of *PRUNE2* on *Akt* pathway activation using *PRUNE2*- and *PCA3*-depleted models.

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APPENDIX

Appendix 1:

Table A1: List of the 86 DEGs in PRUNE2 variant carriers. Genes were considered as significantly deregulated if they had a $|\log_2\text{fold change}| < 1$ and an adjusted p-value < 0.05 . Among the 86 DEGS, 40 were upregulated and 46 downregulated.

Gene Name	log2FoldChange	Adjusted <i>p</i> -value
AC083843,1	1,688743	0,022415
ACAN	-2,399539	0,019952
ADH1B	-1,422565	0,048006
AKR1C2	-2,239161	8,34E-05
ANKRD20A4	-3,299932	0,001587
ASAH2B	-1,891576	0,037864
C4orf47	1,364294	0,038114
C5orf17	4,354743	3,40E-06
CARD10	-1,036386	0,028127
CCDC67	2,505686	0,002829
CCDC83	-4,804485	0,023019
CCL14	-1,814328	0,002829
CCRN4L	1,402172	0,021630
COL28A1	-1,445274	0,029191
COL9A3	-1,988117	0,005749
COMP	-2,671881	0,002114
CPZ	-1,368473	0,003364
CSMD2	-1,590884	0,030958
CTB-89H12,4	1,332656	0,021638
CXCL13	4,436180	0,014712
CXXC5	-1,117993	0,031600
CYP21A2	-1,322489	0,035550
CYP3A5	1,576534	0,038554
CYP4F11	-2,097045	0,032283
DDTL	-3,995738	0,005867
ENG	-1,072123	0,002829
EPSTI1	1,038574	0,004515
FBXO27	2,675751	0,039789
FOSL1	4,151920	0,006372
FOXD3	-3,405046	0,002621
HOXA3	-1,319111	0,007333
HSPA12B	-1,700516	0,048006
IGFBP7	-1,653306	0,002829
KLRB1	1,857520	0,002114
KLRC4	1,407404	0,009555
KRT14	1,385596	0,016331
LAMC2	1,633771	0,022431
LIPG	1,313947	0,008648
MAGI2	1,093868	0,009555
MIDN	1,255858	0,041447

MIR4720	1,319191	0,016240
NABP1	1,138165	0,036199
NALCN	-2,181717	3,49E-05
NELL2	2,066693	0,014377
NRG1	1,663460	0,000748
NRK	1,319286	0,048006
NXPH3	-1,712773	3,40E-06
OR4F21	-5,436241	0,002829
PAGE4	1,223810	0,005430
PCDHA12	-1,760429	0,030795
PCDHGA3	-1,019124	0,035160
PDZD4	-1,057962	0,021764
PGLS	-1,103253	0,004402
PLK1	-1,368648	0,040287
PPFIA2	-1,604501	0,010066
PRG4	-3,542877	0,007590
PTGER3	-1,711786	0,000847
PTPRT	-2,432302	3,93E-04
RASA4	-1,403909	0,016848
RELN	-1,175534	0,035160
RET	-1,628704	0,005138
RGS11	-1,333681	2,47E-04
RP1	3,171856	0,029880
RP11-108P20,1	1,439281	0,030282
RP11-44N11,1	1,048828	0,007280
RP11-553L6,5	1,311327	0,009555
SCNN1G	2,015948	0,012393
SEMG1	-8,795807	0,002044
SEMG2	-6,738636	0,021705
SGCG	1,379733	0,049568
SLC2A5	2,047003	0,013427
SNCA	-1,346113	4,76E-04
SOX12	-1,133845	0,013427
SPAG17	1,415114	0,036206
SPOCK3	1,096795	0,035160
STK17B	1,001380	0,047797
SVEP1	-1,136774	0,010787
SYT13	2,571423	0,023498
THBS4	-1,947103	1,41E-07
TMEM187	-1,202741	0,019952
USP6	3,633730	0,002975
VSTM2A	3,640474	0,030795
WDR49	1,641795	1,11E-05
WDR86	-1,281133	0,030958
ZNF730	1,907639	0,032283
ZNF732	2,196381	0,006259