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# Role of long non-coding RNAs in prostate cancer progression

Rocco Cipolla

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**THÈSE DE DOCTORAT**  
**DE L'UNIVERSITÉ PSL**

Préparée à l'Institut Curie

UMR3244 « Dynamics of genetic information : fundamental bases and cancer »

**Rôle des longs ARN non codants dans la progression du  
cancer de la prostate**

Role of long non-coding RNAs in prostate cancer  
progression

Soutenue par

**Rocco CIPOLLA**

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Composition du jury :

Yves, ALLORY PU-PH, Institut Curie	<i>Président</i>
Eleonora, Leucci Associate Professor, KU Leuven	<i>Rapporteur</i>
Rory, JOHNSON Associate Professor, University College Dublin	<i>Rapporteur</i>
Pablo, NAVARRO DR, Institut Pasteur	<i>Examineur</i>
Han, LI DR, Institut Pasteur	<i>Examineur</i>
Antonin, MORILLON DR, Institut Curie	<i>Directeur de thèse</i>
Marina, PINSKAYA MCU, Sorbonne Université	<i>Co-encadrante</i>



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## **Preface**

The aim of this thesis is to investigate the intricate relationship between long non-coding RNAs and prostate cancer, a prevalent malignancy affecting men worldwide. This introduction provides a comprehensive overview of the key topics that will be explored in this work, namely androgen receptor regulation in prostate cancer, the plasticity of prostate cancer cells, the phenomenon of neuroendocrine transdifferentiation, and the emerging role of lncRNAs in prostate cancer progression and therapeutic response.

# I. INTRODUCTION

## 1. Long non-coding RNAs: from junk RNA to genetic choreographers.

Non-coding RNAs (ncRNAs) were previously considered as transcriptional noise or "junk" with limited functional significance (Clark *et al*, 2011). However, in the recent decades they have emerged as crucial regulators of gene expression and cellular processes, challenging the traditional view of RNA as a mere intermediary between DNA and protein. While still marginal in the perspective of lncRNA's diversity, functional studies in model organisms have provided a growing body of evidence of their involvement in essential biological functions, including embryonic development, cell differentiation and homeostasis, and disease progression.

While protein-coding genes have long been the focus of genetic research, the discovery of ncRNAs has revolutionized our understanding of the complexity of gene regulation and have garnered significant attention due to their intriguing properties and their involvement in a myriad of biological processes.

The history of ncRNAs dates back to the early 20th century when researchers observed RNA molecules that did not code for proteins. However, it was not until the late 20th century that breakthroughs in RNA research, such as the discovery of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), paved the way for understanding the functional diversity of ncRNAs. Key milestones include the identification of small regulatory ncRNAs, such as microRNAs (miRNAs), and the subsequent recognition of long non-coding RNAs (lncRNAs) as significant players in gene expression regulation.

The turning point was with the advent of microarray and high-throughput sequencing technologies that have revolutionized the field, enabling researchers to identify and profile lncRNAs on a genome-wide scale. These efforts revealed that lncRNAs are not mere by-products of pervasive transcription; instead, they constitute a prevalent type of genes with different genomic origins, lengths, and structures. These lncRNAs interact with proteins, DNA, and other RNA molecules within cells, exhibiting regulatory effects at multiple levels.



Understanding the roles and mechanisms of lncRNAs has become a major area of research, and substantial efforts were put into establishing comprehensive databases that annotate lncRNAs in humans, including GENCODE (Frankish *et al*, 2021), RNA Atlas (Lorenzi *et al*, 2021), LNCipedia (Volders *et al*, 2013), FANTOM (Noguchi *et al*, 2017), lncRNAdb (Amaral *et al*, 2011), lncRBase (Das *et al*), NRED (Dinger *et al*, 2009), NONCODE (Zhao *et al*, 2016), and miTranscriptome (Iyer *et al*, 2015). Notably, certain databases, such as Cancer LncRNA Census 2 (CLC2), took a further step, with computational approaches, to integrate functional studies, gene expression, and survival outcomes to identify and predict cancer-associated functional lncRNAs (Vancura *et al*, 2021).

### **1.1. Classification, biogenesis, and properties of lncRNAs.**

lncRNAs represent a diverse class of non-coding RNAs, and their classification is based on various criteria, including genic location, structure, and mode of action. Based on genic location, lncRNAs can be categorized as intergenic, if they are transcribed from regions between annotated genes, or intragenic lncRNAs. The latter can be further divided into a overlapping sense, intronic, antisense, and bidirectional lncRNAs, if they are transcribed from the same promoter as a protein-coding gene, but in opposite direction.

The biogenesis and transcriptional regulation of lncRNAs is akin to that of protein-coding genes and it involves multiple steps, starting with their transcription by RNA polymerase II. Similarly to mRNAs, once transcribed, lncRNA transcripts may be capped at the 5' end with 7-methyl guanosine (m7G), polyadenylated and spliced, generating different isoforms with distinct functions. Post-transcriptional modifications, such as N6-methyladenosine (m6A) methylation (Zhou *et al*, 2017; Yang *et al*, 2018) or pseudouridylation (Zheng *et al*, 2023; Schwartz *et al*, 2014), can also occur and impact lncRNA stability, localization, and interactions with other cellular components. Furthermore, post-transcriptional processing events, such as exonucleolytic trimming (Yin *et al*, 2012), can generate lncRNA isoforms with novel functional properties. For instance, MALAT1 serves as an illustrative example, as it undergoes processing by RNase P and RNase Z, leading to the formation of a 61-nt tRNA-like transcript known as mascRNA (MALAT1-associated small cytoplasmic RNA) (Wilusz *et al*, 2008). Subsequent

studies have demonstrated that mascRNA exhibits an independent mechanism, cooperating with its parent MALAT1, to activate ERK/MAPK signalling in hepatocellular carcinoma cells (Xie *et al*, 2021).

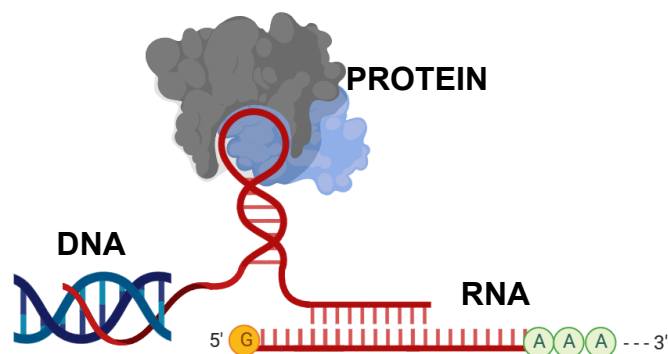
Notably, the biogenesis of lncRNAs is tightly regulated and influenced by various factors, including DNA methylation, histone modifications, and chromatin remodelling. These factors determine the accessibility of the lncRNA loci and their transcriptional activity. This results in a highly specific spatiotemporal expression (Derrien *et al*, 2012; Liu *et al*, 2017), being cell-type and tissue-specific, making lncRNAs rather unique fine-tune regulators of physiological and pathological processes, and optimal disease biomarkers (Pinskaya *et al*, 2019; de Kok *et al*, 2002; Badowski *et al*, 2022).

Once transcribed, mature lncRNAs show remarkable versatility in their intracellular localization. Their distribution in distinct cellular compartments (nucleus and cytosol), membranous and non-membranous organelles (mitochondria, nucleoli, chromatin speckles, paraspeckles, P-bodies, ribosomes), represents a critical aspect of their regulatory functions, finely controlling diverse cellular processes. Moreover, their regulatory reach is extended through their capacity to be secreted outside the cells via extracellular vesicles. Numerous studies have reported the enrichment of these molecules within extracellular vesicles, and some of which have already been implicated in cancer (Almeida *et al*, 2022; Li *et al*, 2019b; Everaert *et al*, 2019). For example, in prostate cancer, the lncRNA *NEAT1* has been found inside exosomes and it has been identified as a critical mediator in the metastatic process. Notably, this lncRNA was found to induce osteogenic differentiation of bone marrow-derived mesenchymal stem cells (hBMSCs) through the activation of Runt-related transcription factor 2 (RUNX2). This is achieved by a two-fold mechanism. First, NEAT1 act as competing endogenous RNA to sponge for *miR-205-5p*, leading to stabilization of RUNX2 mRNA. Secondly, NEAT1 can also promote the IRES-dependent translation of RUNX2 by regulating the Splicing Factor Proline- and Glutamine Rich (SFPQ)/ Polypyrimidine tract-binding protein 2 (PTBP2) complex. (Mo *et al*, 2021).

Although comparative genomics analyses have demonstrated that many lncRNAs are poorly conserved across species (Li & Yang, 2017), recent advancements in the study have revealed that the primary functional unit, and evolutionary constraint, of these molecules is mostly determined by their secondary structure (Johnsson *et al*, 2014). This observation challenges the conventional belief that sequence conservation alone is the

key criterion for assessing the functional importance of lncRNA genes in an evolutionary context. Instead, the focus has shifted towards recognizing other critical features such as structural characteristics and expression patterns from syntenic loci as multidimensional factors that are more relevant and applicable for evolutionary investigations of lncRNAs (Pang *et al*, 2006; Szcześniak *et al*, 2021).

Structurally, lncRNAs exhibit diverse secondary and tertiary structures that determine their functional interactions with other molecules (Figure 1). Their functional repertoire is further expanded by the presence of modular domains often found within their structure, allowing them to engage in multiple interactions simultaneously and facilitate binding to specific proteins and chromatin regions (Graf & Kretz, 2020; Cruz & Westhof, 2009).



**Figure 1. Illustration of types of interaction lncRNAs can engage.**

This structural versatility allows lncRNAs to act as scaffolds, guides, or molecular decoys, dynamically modulating the activities of protein complexes, thereby influencing transcriptional regulation and post-transcriptional processes.

## **1.2. Mechanisms of action of lncRNAs.**

lncRNAs exert their functions through a diverse array of mechanisms mainly thanks to their intrinsic structural features and cellular compartmentalization.

In the nucleus, some lncRNAs recruit chromatin modifiers or remodelling complexes to specific genomic loci, leading to changes in chromatin structure and gene expression. In

this context, they can either act in *cis*, affecting the transcription and/or transcript maturation of neighbouring genes, or far away from their loci, in *trans*.

A well-known *cis* acting lncRNA is *Xist*, which is involved in X-chromosome inactivation in female mammals by recruiting sequentially the transcriptional repressor SPEN (Dossin *et al*, 2020) and the Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) (Brown *et al*, 1991; Borsani *et al*, 1991; da Rocha *et al*, 2014) at the X chromosome.

On the other hand, HOTAIR, represents a milestone *trans*-acting lncRNA with regulatory functions in embryonic development and cancer. It was first characterized by Rin *et al*. in 2007 who were investigating the genomic landscape surrounding the *HOXC* gene cluster. They discovered that HOTAIR is involved in repressing the gene expression by recruiting *in trans* PRC2 and LSD1/REST/coREST complexes at the *HOXD* cluster and other genomic locations (Rinn *et al*, 2007).

LncRNAs can also bind to transcription factors or transcriptional co-regulators, influencing their activities and redirecting their binding preferences. In this context, SLNCR is a notable example of lncRNAs that guides the activity of transcriptional factors. In melanoma cells, for instance, SLNCR mediates the cooperative binding of the androgen receptor (AR) and Brn3a transcription factors to the promoter of the metalloprotease gene MMP9, resulting in its increased expression and consequent gain of invasiveness of melanoma cells (Schmidt *et al*, 2016a). In a different study, the same lncRNA was shown to redirect AR to EGR1-bound genes and mediate the repression of the tumour suppressor CDKN1A/p21, thus promoting proliferation of melanoma cells (Schmidt *et al*, 2019a).

Nuclear lncRNAs are not limited to regulation of gene expression but they can also serve as assembly scaffolds for ribonucleoprotein complexes. For instance, the lncRNA NORAD has been shown to be crucial for the assembly of the NARC1 complex, hence ensuring the correct chromosome segregation and maintenance of genome stability (Munschauer *et al*, 2018). Another example of scaffolding lncRNAs is given by NEAT1, which interacts with NONO and PSF/SFPQ proteins and it is essential for the formation of paraspeckles, non-membranous nuclear structures modulating RNA retention and fate in the nucleus (Clemson *et al*, 2009).

Cytoplasmic lncRNAs act majorly post-transcriptionally via RNA:RNA interactions and also by interfering with protein complexes thanks to their tertiary structures. Some

lncRNAs are localized both in the nucleus and in cytoplasm, highlighting their multifunctional nature. For instance, in the cytoplasm, HOTAIR is known to interact with the E3 ubiquitin ligases Dzip3 and Mex3b through their RNA binding domain and accelerate Ataxin-1 (ATXN1) and Snuportin-1 (SNUPN) ubiquitination and degradation, preventing premature senescence (Yoon *et al*, 2013). Also NORAD, while always acting on the same biological process of the genome stability, does so by folding in a multivalent binding platform and sequestering PUMILIO proteins in the cytoplasm, an example of RNA-driven phase separation (Lee *et al*, 2016).

Moreover, among the protein-interacting lncRNAs, SAMMSON stands as another compelling example due to its significant impact on proteins function through regulation of their subcellular localization. Specifically, SAMMSON promotes cell growth in melanoma cells by orchestrating the interaction between CARF and two proteins, p32 and XRN2. SAMMSON favours the association between CARF and p32 over the CARF-XRN2 interaction. Consequently, p32 is directed towards the mitochondria, whereas XRN2 localize from the nucleoplasm to the nucleoli, resulting in an increase of both cytosolic and mitochondrial translation (Leucci *et al*, 2016; Vendramin *et al*, 2018).

lncRNAs' cytoplasmic functions are not limited to RNA-protein interactions, but they can also pair with other RNA molecules, mRNAs or miRNAs, and impact mRNA splicing, stability, translation, or miRNA-mediated gene silencing. For instance, PTENP1 is a pseudogene-derived lncRNA that exhibits high sequence homology with the functional tumour suppressor PTEN gene but it doesn't code for the protein (Dahia *et al*, 1998). This lncRNA contains multiple binding sites that are complementary to miRNAs known to negatively regulate PTEN expression, thus it acts as competing endogenous RNA (ceRNA) by counteracting miRNA-mediated degradation of the PTEN mRNA (Zhang *et al*, 2017b).

Furthermore, emerging evidence suggests that some lncRNAs contain small open reading frames (sORFs) and can code for functional peptides. LINC00261 is one of such examples of lncRNAs containing a sORF that encodes for a micropeptide, named myoregulin (MLN). MLN is a muscle-specific peptide that inhibits SERCA, a membrane pump of the sarcoplasmic reticulum that controls Ca<sup>++</sup> uptake and skeletal muscle physiology (Anderson *et al*, 2015).

Worth mentioning for its versatility is SLNCR that, while the RNA itself guide transcriptional factors in the nucleus, it also code for a 108 amino acid protein named RASON. In pancreatic ductal adenocarcinoma cells, RASON has been shown to bind KRAS and keep it in an active state by inhibiting both the intrinsic and GAP-mediated GTPase activity, thus promoting proliferation of cancer cells (Cheng *et al*, 2022).

The multifaceted mechanisms of lncRNA action highlight their fascinating versatility in modulating gene expression and cellular functions. They provide intricate layers of regulation that can fine-tune gene expression programs and contribute to the complexity of cellular processes.

However, due to sophistication of their molecular and cellular activities, the functional characterization of the majority of annotated lncRNAs remains an ongoing challenge. As a result, only a small fraction of lncRNAs have been thoroughly studied and their mechanism of action have been elucidated.

### **1.3. Relevance of lncRNAs in biological processes.**

The functional diversity of lncRNAs highlights their relevance in maintaining cellular integrity and regulating intricate biological processes. During development lncRNAs fine-tune key genes and orchestrate precise developmental processes. Some lncRNAs play crucial roles in the lineage commitment, like in the case of *Braveheart*, which it has been shown to interact with PRC2 to mediate epigenetic activation of the cardiac program in mouse embryonic stem cells (mESCs) (Klattenhoff *et al*, 2013). Similarly, lncRNA Tuna (Tcl1 Upstream Neuron-Associated lincRNA) displays an expression pattern restricted to the central nervous system and it is required for the maintenance of pluripotency and neural differentiation in mESCs (Lin *et al*, 2014).

In differentiated cells, lncRNAs are involved in the maintenance of cellular homeostasis. They can participate in cell cycle regulation through modulation of expression of cell cycle genes. ANRIL, for instance, has been proposed to facilitate G1 progression through the recruitment of the PRC2 complex and epigenetic repression in *cis* of P15 within the INK4 locus (Kotake *et al*, 2011). MALAT1 promotes cell cycle progression by acting at both G1 and G2/M transition points by modulating splicing of cell cycle specific transcription factors MYBL2/B-MYB (Tripathi *et al*, 2013). P53-activated lincRNA-p21, instead, leads

to G1 arrest and ensures p53-mediated proapoptotic functions by interacting with hnRNP-K and guiding its genomic localization (Huarte *et al*, 2010).

In the context of tissue regeneration and repair, lncRNAs play vital roles, particularly in processes like wound healing and tissue remodelling. For example, WAKMAR1 have been implicated in these processes. This lncRNA expression is increased during normal wound healing, but it shows reduced levels in non-healing wound. Li *et al*, showed that WAKMAR1 influences the motility of keratinocytes and the reepithelialisation of *ex vivo* skin wounds by interfering with E2F1 promoter methylation and augmenting its expression (Li *et al*, 2019a).

Moreover, lncRNAs are also involved in orchestrating major transcriptomic programs, such as the epithelial-to-mesenchymal transition (EMT). The role of lncRNAs in EMT has garnered significant attention, with several acting as competing endogenous RNAs to promote the transition (HOXA11-AS, lncRNA-ATB, lincROR, lncZNF30-3) (Zhan *et al*, 2018; Yuan *et al*, 2014; Hou *et al*, 2014; Le Hars *et al*, 2023) or scaffolds and guides for chromatin modifying complexes (HOTAIR, HOTTIP, MALAT1) (Jarroux *et al*, 2021; Li *et al*, 2015; Wang *et al*, 2015). Others, act directly on master regulators of EMT. One such example is LETS1, which potentiates TGF- $\beta$  pathway by preventing the SMAD7-mediated polyubiquitination of its receptor, T $\beta$ RI, through the induction of NR4A1, a component of the complex that degrades SMAD7 (Fan *et al*, 2023).

#### **1.4. Relevance of lncRNAs in cancer.**

Given the pleiotropic functions of lncRNAs, it is not surprising that they have been reported to play determining roles in tumour initiation and progression, encompassing all hallmarks of cancer (Gutschner & Diederichs, 2012).

In fact, dysregulated expression of lncRNAs has been observed in various types of cancer, suggesting their involvement in oncogenic processes (Yan *et al*, 2015). Moreover, their cancer- and tissue-specific expression has been proposed to explore clinical applications as diagnostic and prognostic markers or therapeutic targets, providing valuable perspectives in the development of personalized medicine.

LncRNAs have been implicated in multiple aspects of cancer biology and therapy. In cancer initiation, specific lncRNAs can act as oncogenes or tumour suppressors, influencing critical signalling pathways involved in cell proliferation, apoptosis, and DNA damage response. They can promote or suppress malignant transformation by modulating the expression and activity of genes and proteins associated with tumour growth and survival.

In prostate cancer, for example, lncRNA PCAT-1 promotes cell proliferation by stabilizing a known oncogene, cMyc, through the sponging of miR-34a (Prensner *et al*, 2014). With a similar mechanism, PTENP1, mentioned above, exerts oncosuppressive functions by shielding its protein-coding counterpart (Zhang *et al*, 2017b).

During cancer progression, lncRNAs contribute to the remodelling of the tumour microenvironment, angiogenesis, invasion, and immune evasion. A noteworthy example of lncRNA-mediated immune evasion is observed in the case of NKILA lncRNA. Notably, its overexpression in cytotoxic T lymphocytes (CTLs) correlates with CTL apoptosis and reduced patient survival. Additionally, the study revealed that NKILA exerts its effects by inhibiting NF- $\kappa$ B activity, resulting in the sensitization of CTLs within the lung and breast cancer microenvironment to activation-induced cell death (AICD) (Huang *et al*, 2018).

LncRNAs also contribute significantly to the establishment of cancer stem cells (CSCs), which are believed to drive tumour growth and confer therapy resistance. An example of this is lncRNA-Hh, which operates by activating Hedgehog signalling pathway, thereby promoting CSC enrichment and enhancing self-renewal abilities in breast cancer cells (Zhou *et al*, 2016).

Furthermore, lncRNAs have been implicated in the onset of resistance to anticancer therapies, including chemotherapy and targeted therapies. They can modulate drug efflux pumps, alter drug metabolism, or contribute to the rewiring of signalling pathways, resulting in treatment resistance. For example, studies have demonstrated that cancer-associated fibroblasts (CAFs) play a crucial role in supporting chemoresistance and stemness in colorectal cancer cells. This phenomenon is mediated, in part, by secreting exosomal H19, a well-known cancer-associated lncRNA with profound impact on cancer progression (Ren *et al*, 2018). In summary, lncRNAs play critical roles in tumour initiation and progression, acting as oncogenes or tumour suppressors and influencing key



signalling pathways. As our understanding of lncRNAs deepens, their potential therapeutic implications become increasingly compelling. With the ability to modulate the cancer milieu, lncRNAs present themselves as attractive targets for novel diagnostic tools and therapeutic strategies.

Transitioning from the general scope of lncRNAs in cancer, the ensuing chapter will narrow the focus to a more specific context which is the topic of my work. That is prostate cancer and lncRNA involvement to its biology and response to treatment.

## **2. Prostate Cancer: an Overview.**

Prostate cancer (PCa) is characterized by the uncontrolled proliferation of abnormal cells within the prostate tissue. Androgen Receptor (AR) signalling plays a crucial role in the development of prostate cancer, thus androgen deprivation therapy (ADT) and treatments focusing on inhibiting AR have become standard for patients at all stages of the disease. Histologically, the predominant morphology observed in prostate tumours is adenocarcinoma, which closely resembles the regular luminal structure of the prostate. However, as the tumour progresses, an overall disorganization of tissue is observed (Beltran *et al*, 2019). The malignant cells within the adenocarcinoma typically show architectural and cytological features of malignancy, such as loss of glandular architecture, nuclear atypia, increased mitotic activity, and invasion into surrounding tissues. The histological grade of prostate cancer is determined by the degree of architectural differentiation and nuclear abnormalities, commonly assessed using the Gleason grading system (Lotan & Epstein, 2010). In addition to adenocarcinoma, rare

histological variants of prostate cancer can arise, including neuroendocrine tumours, small cell carcinoma, and ductal adenocarcinoma, which exhibit distinct histological characteristics and may have different clinical outcomes (Humphrey *et al*, 2016). Despite the localized prostate cancer having a 5-years survival rate that can be as high as 97%, if detected at early stages, individuals diagnosed with the advanced stage of this disease, known as metastatic castration-resistant prostate cancer (mCRPC), typically exhibit a drop of 5-years survival rate to nearly 30% (Siegel *et al*, 2023). Furthermore, approximately 15-20% of patients with mCRPC undergo a histological transformation from adenocarcinoma to the lethal neuroendocrine subtype, also known as therapy-induced neuroendocrine prostate cancer (t-NEPC) (Abida *et al*, 2019; Beltran *et al*, 2011; Aparicio *et al*, 2011; Yamada & Beltran, 2021).

From the molecular point of view, PCa is associated with specific genetic alterations and biomarkers. For instance, genes like *PTEN*, *TP53*, *TMPRSS2* and *ERG* are commonly found mutated in the majority of prostate cancer cases (Barbieri *et al*, 2012; Abeshouse *et al*, 2015). Also, the overexpression of the prostate-specific antigen (PSA), encoded by the *KLK3* gene, is a common molecular feature of PCa and is often used as a diagnostic marker (Elise Redwine, 1987). Furthermore, as mentioned above, AR is the major driver of the disease progression and mutations or alterations in this specific gene, and many others within the same signalling pathways, are found in most patients (Chen *et al*, 2004; Koivisto *et al*, 1996; Yeh *et al*, 1999).

Besides the well-established protein-coding genes, emerging research has shed light on the involvement of the non-coding genes in PCa development. Many lncRNAs have been found to contribute to PCa development by either acting as oncogenes or oncosuppressors, modulating proliferation, survival, cell invasion, and drug resistance (Huang *et al*, 2019a; Jia *et al*, 2016; Salameh *et al*, 2015; Lingadahalli *et al*, 2018; Gu *et al*, 2019). Noteworthy, PCA3 (Prostate Cancer Antigen 3) has gained attention as a functional lncRNA due to its high specificity of expression in prostate tumour tissues and its enrichment in urines. So far, PCA3 is the only one RNA biomarker approved by the Food and Drug Administration Agency (FDA) in USA and used in clinics for PCa diagnostics (Bussemakers *et al*, 1999; de Kok *et al*, 2002).

## 2.1. Cell types of the prostate gland.

The prostate gland consists of distinct cell types that contribute to its structural and functional complexity. The two main epithelial cell types in the prostate are luminal and basal cells, which are believed to play crucial roles in glandular function and maintenance (Kurita *et al*, 2004, 63; Xin, 2013).

Luminal cells are the predominant cell type in the prostate epithelium, and they are involved in the synthesis and secretion of various proteins and enzymes that contribute to the composition of prostatic fluid. These cells line the glandular ducts and possess specialized features, such as the expression of keratins like cytokeratin 8 (CK8) and cytokeratin 18 (CK18), AR and the secretion of *PSA* (Wang *et al*, 2001).

Basal cells, on the other hand, reside in close proximity to the basement membrane of the prostate epithelium. They serve as a supportive and regenerative population, providing structural integrity to the glandular architecture and acting as progenitor cells for luminal cells. Basal cells express distinct markers, including *P63*, cytokeratin 5 (CK5) and cytokeratin 14 (CK14) (Wang *et al*, 2001).

Besides luminal and basal cells, the prostate gland also contains rare populations of neuroendocrine cells. These cells are scattered throughout the gland and are characterized by the expression of neuroendocrine markers, such as chromogranin A (CHGA), synaptophysin (SYP), and enolase 2 (ENO2) (Di Sant'Agnese, 1998; Sun *et al*, 2009). Neuroendocrine cells can act through paracrine signalling by secreting neuropeptides that are involved in modulating the secretory activity of luminal cells and play a role in regulating prostatic growth and function (Huang *et al*, 2019b; Anthony Di Sant'Agnese, 1992).

The heterogeneous nature of the prostate is further underscored by the existence of a subpopulation of cells known as intermediate cells. Intermediate cells exhibit characteristics of both luminal and basal cells and are thought to possess a transitional state between these two cell types (van Leenders *et al*, 2000).

### **2.1.1. Cellular origins of prostate cancer.**

PCa is a complex disease, and the understanding of its cellular origins has been a topic of research and debate. Traditionally, prostate adenocarcinoma, the most common form of prostate cancer, has been associated with a luminal phenotype. However, the question of whether basal cells, or another cell type in the prostate, can also serve as cells of origin has been raised. Specifically, one hypothesis is that transformed basal cells could differentiate and contribute to the pool of luminal cancer cells.

In experimental models, researchers have observed that transformed human basal cells can give rise to PCa (Wang *et al*, 2006). This has been demonstrated in renal grafting models, where basal cells with genetic alterations have been found to initiate PCa development (Goldstein *et al*, 2010; Stoyanova *et al*, 2013). This suggests that basal cells have the potential to act as cells of origin in PCa.

On the other hand, studies in mice have identified a luminal stem cell population that can serve as a cell of origin for PCa. These studies found that in the regressed mouse prostate, a subset of luminal cells with stem cell properties can initiate prostate cancer formation (Wang *et al*, 2009).

In other experiments involving the deletion of the oncosuppressor PTEN in either basal or luminal cells have shown that both cell types can act as cells of origin for PCa (Choi *et al*, 2012; Lu *et al*, 2013; Korsten *et al*, 2009), suggesting that prostate cancer can arise from both basal or luminal cells in the presence of specific genetic alterations.

However, more recent research using new lineage tracing techniques in mice has provided additional insights into the cellular origins of prostate cancer, and allowed the authors to conclude that luminal cells are consistently favoured as cells of origin for PCa (Wang *et al*, 2014).

### **2.2 Role of androgens and Androgen Receptor in prostate cancer.**

Androgens, such as testosterone and dihydrotestosterone (DHT), along with their receptor, AR, play a critical role in prostate cancer development and progression. In the normal prostate gland, the activation of AR involves complex molecular mechanisms. One primary mechanism is the binding of androgens, which initiates a series of events leading

to its activation. Upon ligand binding, AR is released from the heat-shock proteins HSP70 and HSP90 and undergoes conformational changes that allows its dimerization and further activation by phosphorylation thanks to the recruitments of kinases (Tan *et al*, 2015b). This facilitates its translocation from the cytoplasm into the nucleus. In the nucleus, the AR binds to androgen response elements (AREs) in the DNA, resulting in the recruitment of co-activators, chromatin remodelling complexes, and other components of the transcriptional machinery (Heemers & Tindall, 2007; Yu *et al*, 2010). This ultimately leads to the activation of target genes involved in cell growth, proliferation, survival, and other processes critical for prostate gland function.

While the AR is widely recognized for its role as a transcriptional activator, it can also act as a transcriptional repressor through the association with repressive cofactors and complexes such as PRC2 and REST/coREST/LSD1 (Zhao *et al*, 2012a; Cai *et al*, 2014). This dual capability significantly broadens the impact AR can have on gene expression regulation.

However, in PCa, various genetic and epigenetic changes can disrupt normal androgen signalling, leading to an increased reliance on androgens for tumour growth. This is often accompanied with the overexpression of AR in mCRPC tumours. Nevertheless, not all mCRPC tumours exhibit this overexpression, indicating the existence of additional mechanisms, independent of AR, responsible for castration resistance (Edwards *et al*, 2003).

### **2.3. Androgen deprivation and Androgen Receptor-targeted therapies.**

The purpose of ADT is to reduce or block the activity of androgens in patients, and it is typically achieved through surgical or medical castration, such as orchiectomy and use of gonadotropin-releasing hormone agonists (Harris *et al*, 2009). However, the therapeutic potential of inhibiting both the androgen production and AR ligand binding led to the development of AR antagonists. Therapies with AR inhibitors (ARIs) have revolutionized the management of PCa. These therapies aim to inhibit AR signalling and its downstream effects on tumour growth. Anti-androgens, such as enzalutamide and apalutamide, competitively bind to the ligand-binding domain of the AR, thus inhibiting receptor activation (Scher *et al*, 2008; Clegg *et al*, 2012). Another approach is the use of

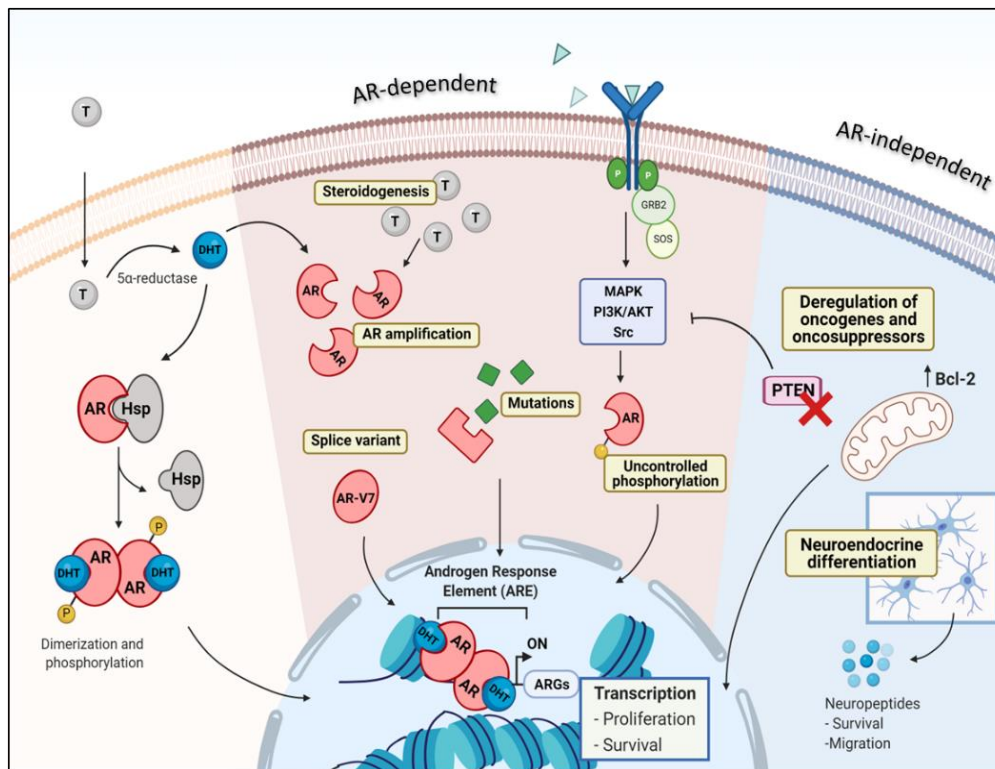
abiraterone, which reduce androgen production by blocking the *CYP17* enzyme responsible for androgen synthesis in the adrenal glands and the tumour microenvironment (Reid *et al*, 2008).

#### **2.4. Progression to castration-resistant prostate cancer.**

While initially effective, resistance to AR-targeted therapies can emerge, leading to disease progression to castration-resistant prostate cancer (CRPC). Although the majority of CRPC are adenocarcinoma with active AR signalling, emerging epidemiological studies have reported a significant proportion of CRPC tumours that have lost AR dependence (Shah *et al*, 2004). In addition, CRPC tumours display a remarkable heterogeneity as they manifest a wide-range of phenotypes: while some AR-null tumours express neuroendocrine (NE) markers (e.g., small cell neuroendocrine prostate cancer, SCNPC) or they are identified as double negative for AR activity and NE markers (DNPC) (Bluemn *et al*, 2017), others exhibit a mixed phenotype of NE features coexisting with AR activity (Aggarwal *et al*, 2018). Interestingly, Labrecque *et al.*, by coupling histological and transcriptomic data, identified five different phenotypes in metastatic CRPC based on AR activity and neuroendocrine signature (Labrecque *et al*, 2019): adenocarcinoma with high AR activity (ARPC), adenocarcinoma with heterogenous and low expression of AR (AR low PC, ARLPC); samples with concomitant AR activity and NE signature (amphicrine PC, AMPC); small cell neuroendocrine carcinoma with NE markers and lacking AR expression (SCNPC); finally, tumours lacking both phenotypes, named double-negative PC (DNPC). Overall, the emergence of these heterogenous phenotypes in CRPC highlights the need for further research to better understand the complexity of disease progression and treatment resistance.

## 2.5. Mechanisms of resistance to androgen deprivation therapy.

Several resistance mechanisms have been identified, that can span from aberrant AR signalling to neuroendocrine transdifferentiation (NED) of PCa cells (Figure 2). Understanding these resistance mechanisms is crucial for the development of novel therapeutic strategies to improve outcomes for patients with advanced disease.



**Figure 2. AR-dependent and -independent mechanisms of resistance to therapy.**

At physiological levels, testosterone (T) is converted into dihydrotestosterone (DHT) which then binds and leads to the activation of AR through dimerization and phosphorylation. Among the AR-dependent mechanisms, intracellular production of T compensates the low levels of T post-ADT. Mutations and splice variants of AR allow the activation of the transcriptional factor in the absence of its ligand. Uncontrolled phosphorylation by Src family kinases bypasses the need of ligand binding and enables AR transcriptional activation. Also, AR-independent mechanisms such as deregulation of oncogenes and oncosuppressors facilitate the progression to CRPC. Neuroendocrine transdifferentiation of PCa cells is often associated with the onset of CRPC.

### 2.5.1. Androgen Receptor- and androgen-dependent mechanisms.

A common genetic perturbation that affects AR signalling is the AR gene amplification, observed in a subset of prostate cancer cases (Koivisto *et al*, 1996), which

can result in an increased number of AR copies within tumour cells, thereby maintaining AR signalling.

Point mutations in the AR gene can alter the receptor's structure and function, rendering more sensitive its binding to androgen and non-canonical ligands (Sack *et al*, 2001; Li *et al*, 2008), such as progesterone and glucocorticoid hormones, or even enabling constitutive activation in the absence of androgens (Céraline *et al*, 2004).

In addition to mutations, the expression of alternative splice variants can give rise to truncated AR proteins that bypass the androgen deprivation. Among these, the most commonly found in CRPC are AR-V7, that contains the cryptic exon 3 (CE3) present within the canonical intron 3, and AR<sup>v567es</sup>, that misses exons 5-6-7 (Hu *et al*, 2009; Sun *et al*, 2010). Both these variants produce AR proteins with the truncated C-terminal ligand-binding domain that results in the constitutive activation of AR in a ligand-independent manner.

Normally, androgen production mainly occurs in the testis and adrenal glands. When this is blocked by ADT, many PCa cells undergo adaptation that enables them to produce androgens themselves and sustain tumour growth. In fact, despite the drastic decrease of testosterone levels, in up to 90% of ADT-treated tumours, the activation of AR persists, due to the intracrine production of the hormone by cancer cells (Titus *et al*, 2005). In this context, cancer cells can newly synthesize the 5 $\alpha$ -dihydrotestosterone (DHT), a more potent activator than testosterone, by using circulating steroid precursors such as progesterone and cholesterol (Dillard *et al*, 2008).

Moreover, ADT and AR-targeting therapies have been shown to release the transcriptional repression of AR, enabling expression of NE-related genes and driving the NED transcriptional program (Kregel *et al*, 2013; Bishop *et al*, 2017; Akamatsu *et al*, 2015).

### **2.5.2. Androgen Receptor- and androgen-independent mechanisms.**

Alongside resistance mechanisms centred around androgens and AR activity, there are several alternative pathways and processes that operate independently of AR and contribute to treatment failure, as well as progression to CRPC.



These mechanisms are known as *bypass pathways* and play a significant role by providing growth and survival signals. Bypass pathways often utilize receptor tyrosine kinases (RTKs) to activate a variety of kinases, such as MAPK/Ras/Raf (Bluemn *et al*, 2017), which then trigger the activation of different transcription factors like NF- $\kappa$ B and c-MYC (Bakin *et al*, 2003; Gioeli *et al*, 1999; Mukherjee *et al*, 2011). These transcription factors can induce changes that impact cell cycle regulation and proliferative properties of cells (Jin *et al*, 2014). One crucial signalling cascade that is altered in advanced CRPC is the Akt pathway (Cham *et al*, 2021). Akt signalling can function both in collaboration with AR or independently through intermediate proteins that influence significant cellular processes, including apoptosis and proliferation in PCa cells (Xin *et al*, 2006).

Additionally, in some tumours the glucocorticoid receptors (GR) can take up the role and cross-activate AR target genes. Both AR and GR belong to the same class of nuclear steroid receptors with high similarities in structure, specifically in the DNA-binding domain (DBD), and in the mechanism of action (Hu & Funder, 2006; Mangelsdorf *et al*, 1995). Consequently, both receptors share similar genomic targets, and in a study from Arora *et al*. it has been showed that the overlap between the two regulomes was sufficient to confer resistance to enzalutamide, whereas GR antagonists could restore the sensitivity to treatment (Arora *et al*, 2013).

Furthermore, the phenomenon of NED, where PCa cells acquire a neuroendocrine phenotype and lose their dependence on AR signalling, can contribute to therapy resistance. While only a small percentage (0.5-2%) (Helpap *et al*, 1999) of primary PCa are initially diagnosed as neuroendocrine prostate cancer (NEPC), a significant proportion, up to 20% (Abida *et al*, 2019; Beltran *et al*, 2011; Aparicio *et al*, 2011), of mCRPC acquires NEPC characteristics. The transition from PCa adenocarcinoma to NEPC has gained recognition as a crucial mechanism of treatment resistance, leading to the development of an exceptionally aggressive PCa subtype. This remarkable shift in phenotype, triggered by the selective pressure exerted by ADT, is part of the lineage plasticity of PCa cells, and highlights the ability of PCa cells to adopt different cellular lineages in response to therapeutic pressures. The transition towards NEPC involves recurring genetic changes that encompass mutations or deletions in RB1 and TP53, alongside the overexpression and genomic amplification of MYCN and AURKA (Beltran *et al*, 2016). NEPCs also exhibit genetic abnormalities commonly found in prostate

adenocarcinomas, such as ETS (E26 transformation specific) rearrangements (Williamson *et al*, 2011; Lotan *et al*, 2011). These joint genetic abnormalities support the hypothesis of a shared origin between NEPC and prostate adenocarcinomas, implying an interconnected evolutionary relationship between these cancer subtypes.

## **2.6. LncRNAs in prostate cancer progression and resistance to therapy.**

The landscape of lncRNAs associated with prostate cancer is vast, encompassing numerous identified candidates. However, despite their abundance, only a small fraction has been comprehensively investigated from a mechanistic point of view, and few have been associated with CRPC and NEPC acquisition (Ramnarine *et al*, 2019).

One such lncRNA is CCAT1, which has been identified as an oncogenic lncRNA in a variety of tumors (Nissan *et al*, 2012; Dou *et al*, 2017; Zhang *et al*, 2017a). Notably, CCAT1 is upregulated in CRPC, and its expression levels have been found to correlate with patient mortality. In vitro studies have demonstrated that CCAT1 promotes proliferation and colony formation while suppressing apoptosis. Mechanistically, CCAT1 acts as ceRNA for the tumour suppressive miRNA, miR-28-5p, in the cytoplasm. While in the nucleus, CCAT1 acts as scaffold for DDX5/P68 and AR, reinforcing the expression of androgen responsive genes (You *et al*, 2019).

HOTAIR is another example of lncRNA that increases the AR transcriptional activity in CRPC. This is achieved through preventing the polyubiquitination of AR by blocking its binding to the E3 ubiquitin ligase MDM2 (Zhang *et al*, 2015a).

Additionally, PCAT1 has been implicated in CRPC progression by regulating the PHLPP/FKBP51/IKK $\alpha$  complex. Specifically, PCAT1 binds directly FKBP51 and displaces PHLPP from the complex, leading to the activation of AKT and NF- $\kappa$ B signalling (Shang *et al*, 2019).

MALAT1 exhibits a broad range of effects on prostate cancer cells, with implications in tumour growth, invasion, and metastasis formation (Ren *et al*, 2013). However, the precise underlying molecular mechanisms governing these effects remain largely unexplored. In a specific study, it was proposed that MALAT1 enhances EZH2-mediated CRPC cells invasion through direct binding to its N-terminal region (Wang *et al*, 2015).

The aforementioned LINC00261, encoding the MLN micropeptide with a regulatory activity in the Ca<sup>++</sup> uptake (Anderson *et al*, 2015) in muscles, was also shown to contribute to proliferation and metastasis in NEPC. In PCa, its activity in PCa relies on the lncRNA itself using two distinct mechanisms (Mather *et al*, 2021). In the nucleus, it was proposed to guide SMAD2/3 transcription factors to the *FOX2A* promoter *in cis* and activate its transcription, while in the cytoplasm, LINC00261 plays a role of competing endogenous lncRNA counteracting miR-8485 targeting of CBX2 mRNA. However, the LINC00261-derived peptide contribution to NEPC has not been investigated yet.

Addressing therapeutic resistance, the lncRNA PCDRLnc1 confers docetaxel resistance to PCa cells by promoting autophagy. Its mechanistic involvement lies in its interaction with UHRF1 and subsequent activation of Beclin-1 autophagic signalling (Xie *et al*, 2022).

Likewise, the lncRNA PCBP1-AS1 has been identified as a mediator of enzalutamide resistance. By stabilizing the USP22-AR complex, it promotes deubiquitination of AR, thus leading to the maintenance of AR signalling regardless enzalutamide resistance. Notably, knockdown of the lncRNA restores cells sensitivity to enzalutamide (Zhang *et al*, 2021).

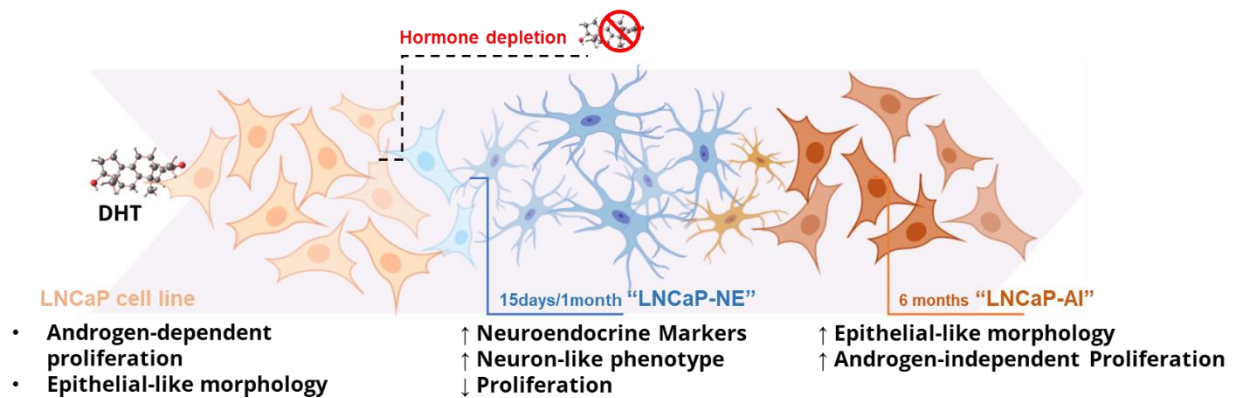
It is noteworthy to mention the role of the lncRNA-p21 in resistance to treatment and NED. Recent findings reported its elevated expression in NEPC samples and showed that this lncRNA promotes NED in enzalutamide treated PCa cells. Specifically, lncRNA-p21 disrupts EZH2 interaction with other components of the PRC2 complex, and facilitate its association with STAT3, triggering STAT3 activation and subsequent NED (Luo *et al*, 2019). Similarly, lncRNA H19, upregulated by SOX2 after hormone deprivation, is pivotal in modulating cellular plasticity and resistance to therapy. Its knockdown promotes a luminal phenotype and re-sensitize PCa cells to ADT, whereas its expression pushes towards a NE phenotype. At the mechanistic level, H19 coordinates the PRC2 complex, influencing histone H3 methylation levels of both AR-driven and NEPC-related genes (Singh *et al*, 2021).

In the intricate landscape of molecular mechanisms underlying prostate cancer, lncRNAs have emerged as pivotal elements, especially within the challenging milieu of CRPC. Their diverse regulatory functions, spanning from regulation of gene expression to peptide-coding, highlights their importance in cancer biology. However, a significant portion of the lncRNAs identified remains largely unexplored. While substantial research has

spotlighted a few lncRNAs in the context of CRPC, the critical transition to NEPC sees only a handful of these molecules thoroughly investigated. Bridging this knowledge gap is essential not only for understanding disease progression but also for uncovering potential therapeutic paths. Given this context, this thesis seeks to explore in-depth into the complex roles of lncRNAs, focusing on their specific contributions to the development of CRPC and, specifically, to the emergence and dynamics of NEPC.

### 3. Objective of the thesis.

The objective of this project is to investigate the transcriptomic changes that occur in response to androgen deprivation and NED, with a particular focus on the regulation of AR signalling pathway and the onset of CRPC. For this purpose, as described in figure 3, we employed a dynamic *in vitro* cell system, LNCaP, able to recapitulate NE and androgen-independent phenotypes upon androgen deprivation (Terry *et al*, 2013a). Over a six months period following hormone (DHT) depletion, LNCaP cells exhibit substantial and progressive phenotypic changes. The most notable alterations occur at the one-month mark (NE-1m, neuroendocrine-like 1 month), where cells display neuroendocrine features and a neuron-like morphology. By six months (AI, androgen-independent), the cells revert to an epithelial-like morphology and regain the ability to proliferate even in the absence of DHT.



**Figure 3. Illustration of dynamic *in vitro* cell model of response to hormone depletion.**

Within this framework, we set two objectives. The primary aim is to investigate the transcriptome changes associated with this dynamicity, aiming to identify and characterize lncRNAs involved with the response to androgen deprivation and their respective mechanisms. This will constitute the major body of the thesis and will be discussed in the first part of the Results section. Our secondary focus lies in investigating the activity of AR, and its consequence on epigenetic landscape reconfiguration, underlying cellular plasticity during NED by assessment of AR subcellular localization, expression of its target genes, and its chromatin binding in relation to different genomic elements and histone modifications. This will be discussed in the second part of the thesis.

## II. Results

### 1. A novel lncRNA PROCA11 regulates cell plasticity in response to androgen deprivation of prostate cancer cells.

The emerging paradigm of lncRNAs as pivotal drivers of prostate cancer progression and resistance to therapeutic interventions prompted us to perform a deeper exploration of this intricate transcriptomic landscape, specifically targeting the molecular underpinnings of therapy-induced neuroendocrine differentiation and subsequent androgen independence following androgen deprivation.

Relying on reported LNCaP *in vitro* cellular model's capacity to recapitulate the neuroendocrine transdifferentiation and acquisition of androgen-independence, we mapped dynamic transcriptomic alterations associated with the process and, through rigorous bioinformatics analysis, identified novel lncRNA players elicited by the androgen deprivation.

Notably, through the integration of de novo transcriptome assembly and differential expression analysis of RNA-sequencing performed at different time points of the transition, we identified 15 novel and 4 already annotated lncRNAs, hereby designated as PROCAs (PROstate Cancer Associated lncRNAs), activated in response to androgen deprivation and associated with acquisition of neuroendocrine traits. Among these candidates, our attention was drawn towards PROCA11, a novel non-coding transcript exhibiting substantial expression in both LNCaP and advanced prostate cancers. Strikingly, PROCA11 displayed an intricate regulatory relationship with the AR activity, modulating cell proliferation and cell cycle of cells.

The findings encapsulated in the ensuing publication offer a functional characterization of this novel lncRNA in prostate cancer cells.

# A novel lncRNA PROCA11 regulates cell plasticity in response to androgen deprivation of prostate cancer cells

Rocco Cipolla<sup>1</sup>, Marc Gabriel<sup>1</sup>, Micaela Piemontese<sup>1</sup>, Giorgia Ianese Regin<sup>1</sup>, Ugo Szachnowski<sup>1</sup>, Virginie Firlej<sup>2</sup>, and Marina Pinskaya<sup>1,\$</sup> & Antonin Morillon<sup>1,\$</sup>

<sup>1</sup> ncRNA, Epigenetic and Genome Fluidity, CNRS UMR3244, Sorbonne Universités, PSL University, Institut Curie, Centre de Recherche, Paris, France

<sup>2</sup> TRePCa, Université Paris Est Créteil, Créteil, France

<sup>\$</sup> co-corresponding and co-last authors

**Keywords:** castration-resistant prostate cancer (CRPC), neuroendocrine prostate cancer (NEPC), lncRNA, Androgen Receptor (AR), androgen deprivation therapy (ADT)

## Abstract

Long non-coding RNAs (lncRNAs) represent vast and yet poorly characterized family of genes that can fine tune cellular plasticity, thereby allowing the emergence of aggressive therapy-resistant and metastatic cancers. Androgen deprivation therapies (ADT) are commonly used to treat prostate cancer by inactivating the Androgen Receptor (AR). However, castration-resistant prostate cancer (CRPC) with neuroendocrine subtypes (NEPC) often emerge. In this study, we explore the role of lncRNAs in response to androgen deprivation. Using a dynamic prostate cancer cell system mimicking the CRPC and NEPC onset, we identified 15 novel lncRNAs, with PROCA11 standing out as a first-choice candidate, being also highly abundant in high-risk prostate cancer tumors. This majorly nuclear lncRNA is expressed at low levels in androgen-dependent conditions of growth and strongly activated upon hormone withdrawal, preceding neuroendocrine genes and persisting at high levels in neuroendocrine cells. Extensive computational analysis of clinical data and functional studies in cells revealed PROCA11 association with basal-to-luminal transformation of the transcriptomic landscape and activation of metabolic and signaling pathways reminiscent of neurogenesis and of maintenance of AR signaling. We propose that PROCA11 is involved in the intricate circuits regulating cellular plasticity enabling cell survival and proliferation and emergence of the NE phenotype in response to ADT.

## Introduction

Initiation and progression of prostate cancer depend on androgens and on the androgen-inducible transcription factor - Androgen Receptor (AR). Hence, androgen deprivation therapies (ADT) in combination with AR antagonists are used as a first-line treatment of prostate cancer. However, patients often relapse and develop castration-resistant prostate cancer (CRPC), dropping the 5-years survival rate to 30% (Siegel *et al*, 2023). Furthermore, up to 20% of CRPC patients undergo histological transformation from adenocarcinoma to neuroendocrine subtype, also known as therapy-induced neuroendocrine prostate cancer (tNEPC), which is considered lethal (Beltran *et al*, 2011); (Abida *et al*, 2019); (Yamada & Beltran, 2021). CRPC is characterized by high heterogeneity in tissue morphology, cellular and molecular characteristics. Emerging single-cell and bulk transcriptomic studies of post-ADT tumors reveal high heterogeneity with a complex combination of cell types for majority retaining properties of prostate adenocarcinoma

with still active AR signaling, but some progressively acquiring androgen independence (AI) and/or undergoing neuroendocrine (NE) trans-differentiation giving rise to tNEPC (Patel *et al*, 2019); (Beltran *et al*, 2019); (Beltran *et al*, 2012); (Labrecque *et al*, 2019); (Risbridger *et al*, 2021). However, these trajectories are not linear and prevailing, reflecting high lineage plasticity of advanced prostate cancer tumors due to multiple genetic alterations and activation of numerous reprogramming transcription factors (ONECUT2, BRN2, SOX2, OCT4, ASCL1) and chromatin modifiers (EZH2, SWI/SNF) in response to inhibition of the AR signaling (Choi *et al*, 2022); (Han *et al*, 2022); (Nouruzi *et al*, 2022); (Bishop *et al*, 2017); (Cyrta *et al*, 2020).

Recently massive RNA-sequencing revealed thousands of long noncoding (lnc)RNAs, constituting a major family of genes in humans, highly expressed in CRPC and some in NEPC tumors (Abida *et al*, 2019); (Ramnarine *et al*, 2018); (Iyer *et al*, 2015). Among them, MIAT, lncRNA-p21, LINC00261 and H19 were described as regulators of the NE trans-differentiation in prostate cancer (Crea *et al*, 2014); (Luo *et al*, 2019); (Singh *et al*, 2021); (Mather *et al*, 2021). These lncRNAs were shown to contribute to tumor plasticity and cancer progression modulating gene expression patterns mostly at the transcriptional level. MIAT, a myocardial infarction associated transcript, and H19 are up-regulated in NEPC and proposed to interact with and regulate the activity of PRC2 reshaping H3K27me and H3K4me3 at gene promoters towards neuroendocrine trans-differentiation (Crea *et al*, 2014); (Singh *et al*, 2021). Another study revealed that the treatment of CRPC patients with the AR antagonist enzalutamide induces the expression of lncRNA-p21 enabling switching the EZH2 methyltransferase activity from the histone H3K27 to a non-histone substrate, the STAT3 transcription factor (Luo *et al*, 2019). Finally, LINC00261 is an example of an evolutionary conserved lncRNA, which contributes to proliferation and metastasis in NEPC through two distinct nuclear and cytoplasmic mechanisms (Mather *et al*, 2021). Apart from these unique examples, our knowledge of how lncRNAs enable to circumvent the chemical castration in the onset of CRPC and NEPC stays very limited.

In this study we took advantage of the dynamic prostate cancer cell system recapitulating the CRPC onset to identify lncRNAs induced early in response to androgen deprivation. Total RNA-sequencing followed by differential expression analysis of *scallop*-assembled transcripts and multiple filtering isolated 15 novel lncRNAs, including the top candidate PROCA11, which are highly expressed in high-risk prostate cancer tumors and associated with emergence of NEPC traits. This majorly nuclear lncRNA, presenting 3 main isoforms, is lowly expressed in hormone-dependent cells but activated following AR knock-down or due to a release of AR from *PROCA11* promoter upon androgen deprivation. Activation of *PROCA11* transcription precedes those of NE genes and persists at high levels though NE trans-differentiation. Gain- and loss-of-function experiments demonstrate that *PROCA11* promotes cell proliferation regulating a balance between cAMP and RAS signaling, synaptic and metabolic activities. We propose that in the context of androgen deprivation of prostate cancer cells, *PROCA11* can modulate metabolic profile and potentiate cells for survival and proliferation in the route of acquisition of neuroendocrine phenotype.

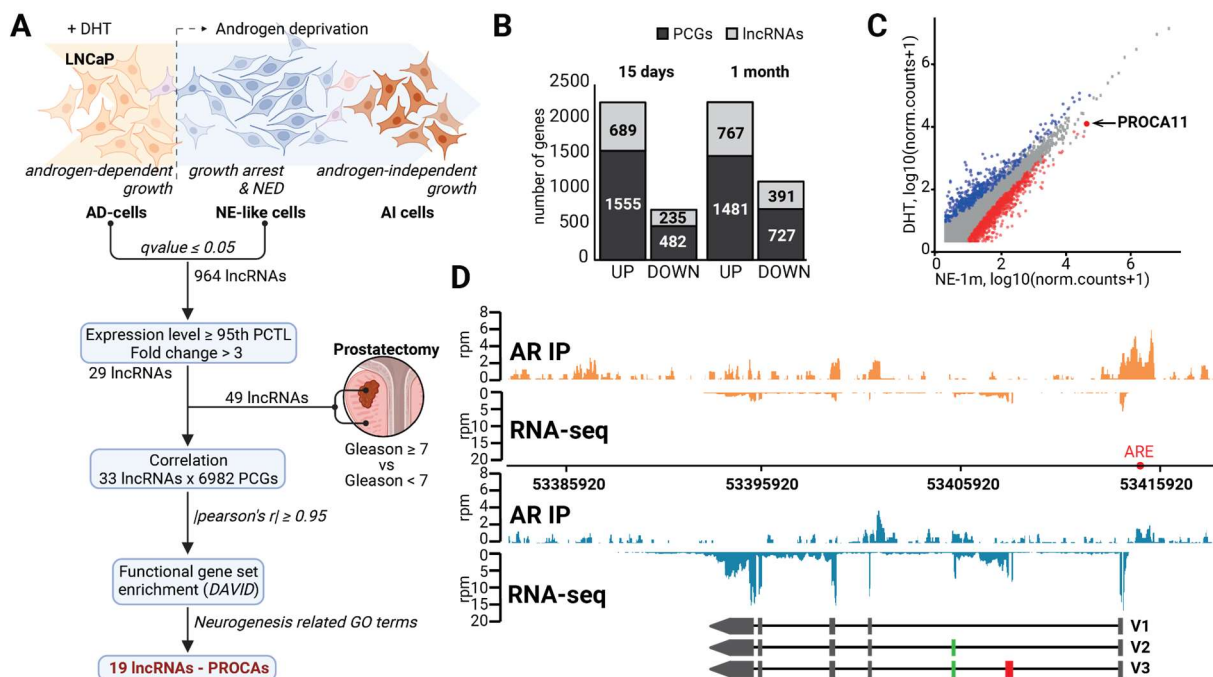
## Results

### Discovery of a novel lncRNA PROCA11, induced by androgen deprivation of prostate cancer cells

To characterize transcriptome changes underlying cell plasticity in response to androgen deprivation we took advantage of a dynamic *in vitro* cell model recapitulating early response to ADT androgen deprivation and progressive acquisition of NE-like and AI traits (Terry *et al*, 2013).

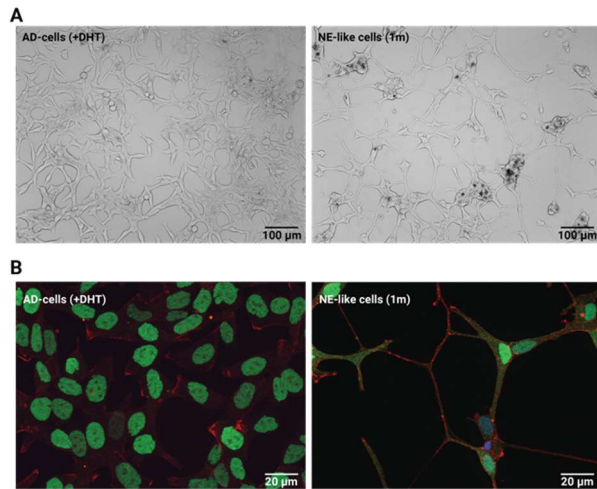


In this system, first, adenocarcinoma LNCaP cells are grown in the presence of dihydrotestosterone (DHT) mimicking primary epithelial androgen-dependent luminal tumors (AD-cells). Then, they are subjected to androgen deprivation which induces immediate growth arrest, progressive change in cell morphology and properties towards the neuroendocrine-like phenotype (NE-like cells) with weaker but still persisting AR in the nucleus (Figure 1A; Supplementary Figure 1A-B). We performed a total stranded RNA-sequencing of these cell populations at different time points of transition and quantified gene expression using a custom transcriptome annotation including the Gencode release 32 and *scallop*-assembled transcripts from intergenic and antisense regions and regions overlapping annotated noncoding sense exonic transcripts (see Material and Methods). To focus on the earliest changes induced by androgen deprivation we further performed a differential expression analysis comparing two conditions: AD-cells actively proliferating in the presence of DHT and growth arrested NE-like cells cultured for 15 days and 1 month in hormone-depleted media (NE-15d/1m) (Figure 1A). In total, 964 lncRNAs were significantly induced by androgen deprivation (adj. p-value  $\leq 0.05$ , Figure 1B). Further selection of the most robust lncRNA candidates was done through a three-fold filtering: (i) normalized read counts above 95<sup>th</sup> percentile in NE-15d together with (ii) a significant increase of expression in high-risk versus low-risk prostate tumors of prostatectomy origin (adj. p-value  $\leq 0.05$ ) (Pinskaya *et al*, 2019); and (iii) co-expression with protein-coding genes functionally linked to neurogenesis (Pearson's  $r \geq 0.99$ ) (Figure 1B). This allowed isolation of four already annotated and fifteen novel lncRNAs with the top most up-regulated candidate, named PRostate Cancer lncRNA 11 (PROCA11) (Figure 1C).



**Figure 1.** Discovery and characterization of novel PRostate Cancer Associated (PROCA) lncRNAs, expressed in response to androgen deprivation: (A) Experimental and bioinformatics workflow for discovery of lncRNAs associated with the onset of the NE phenotype (created with BioRender.com); (B) Significantly deregulated protein-coding (PCGs) and lncRNA genes identified in NE-like cells after 15 days and 1 month of androgen deprivation (adj. p-value  $\leq 0.05$ ); (C) 2D-plot of gene expression in  $\log_{10}(\text{normalized counts})$  showing down-regulated genes (blue) and up-regulated genes (red), including PROCA11, upon 1 month of androgen deprivation; (D) PROCA11 RNA-seq and AR CUT&RUN profiling in AD- (orange) and NE-1m (blue) cells across the chromosome 8 region embedding a newly annotated gene,

genomic position of the putative ARE (GGACAAGGA) is indicated as a red dot. PROCA11 isoforms V1, V2, and V3 identified by molecular cloning and Sanger sequencing are represented with exons shown as rectangles, and introns as lines.

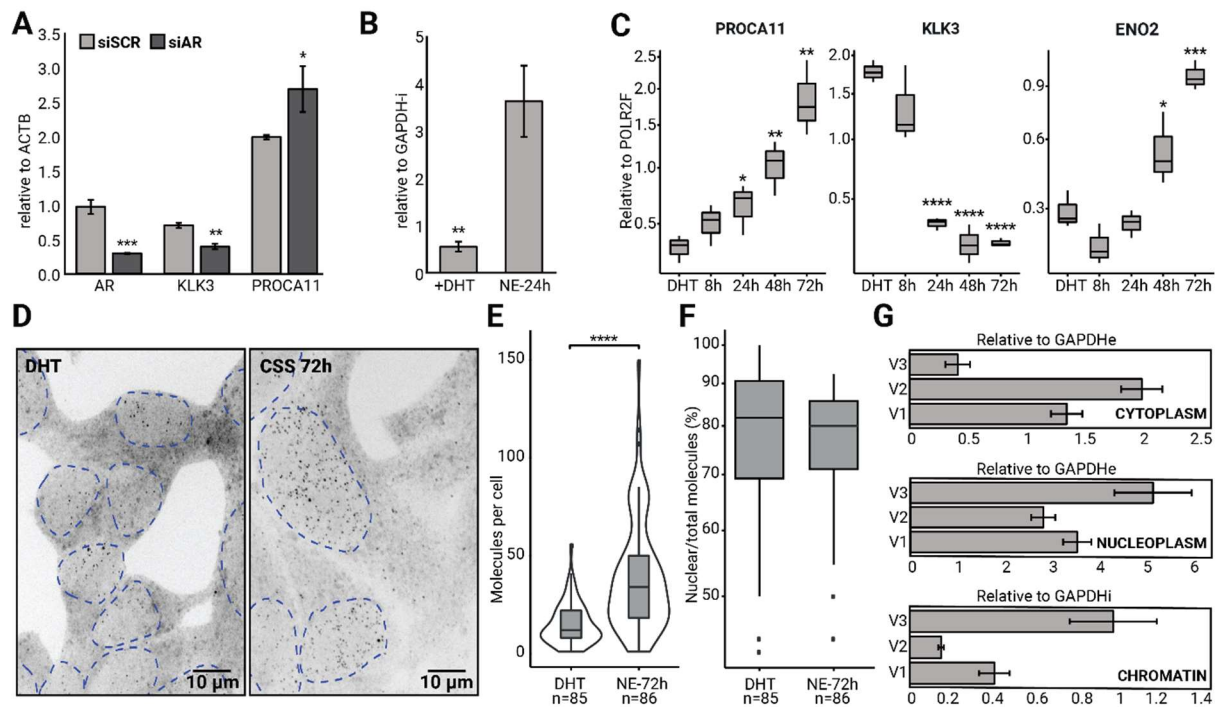


**Supplementary Figure 1.** Androgen deprivation induces changes in cell morphology and AR subcellular localization:

(A) Bright-field inverted microscope images of LNCaP cells growing in the presence of DHT (AD-cells) and in the absence of hormone for one month (NE-like cells, NE-1m); (B) Immunofluorescence imaging of AR (green), F-actin stained by phalloidin (red) and DNA stained by DAPI (blue) in AD- and NE-1m cells.

Molecular cloning and sequencing identified three major isoforms of PROCA11, differing in inclusion of the second and third exons, that we named PROCA11 V1, V2 and V3, respectively (Figure 1E). Since PROCA11 expression was low in AD-cells but strongly stimulated in NE-like cells, we hypothesized that its transcription can be regulated by AR. Indeed, CUT&RUN profiling revealed a presence of AR at the *PROCA11* promoter, enclosing an *in silico* predicted androgen response element (ARE) (Messegueur *et al*, 2002), in AD-cells growing in the presence of androgen. The AR peak was dramatically decreased upon androgen deprivation concomitantly with the increase in PROCA11 levels (Figure 1D).

To understand the relationship between AR and PROCA11 expression we performed RNAi-mediated depletion of AR in AD-cells. It resulted in a 30% increase of PROCA11 levels in contrast to the prostate specific antigen (PSA)-encoding *KLK3* gene, a known AR-activated target (Figure 2A). We, next, isolated nascent transcripts from the chromatin fraction of cells treated with alpha-amanitin to block transcription. Further quantification of intronic sequences revealed a substantial increase of PROCA11 nascent transcript levels and, hence, of its transcription upon androgen deprivation (Figure 2B). This response was very rapid, even preceding the detection of the characteristic NE marker - ENO2 (Figure 2C). Finally, we assessed a subcellular localization of PROCA11 by single-molecule RNA-FISH (smiFISH). In cells growing in the presence of the DHT hormone we detected in average 15 molecules of PROCA11 per cell increasing up to 2.5-fold upon hormone withdrawal for 72 hours (Figure 2D-E). In both conditions up to 80% of PROCA11 transcripts was localized in the nucleus (Figure 2F). Albeit, subcellular fractionation and quantification of specific PROCA11 isoforms revealed their uneven distribution with V1 and V2 being more abundant in the cytoplasm and V3 in the chromatin (Figure 2G).



**Figure 2.** PROCA11 is an AR-repressed lncRNA transcriptionally activated very early upon androgen deprivation and localized majorly in the nucleus: (A) RTqPCR quantification of PROCA11 lncRNA, AR, and KLK3 levels in cells treated with siAR or siSCR 24 hours post-transfection, relative to siSCR; (B) RTqPCR quantification of nascent PROCA11 as intron relative to GAPDH intron in the chromatin fraction of alpha-amanitin treated cells growing in the presence or absence of DHT for 72h; (C) RTqPCR quantification of PROCA11, KLK3, and of the NE marker ENO2 in AD-cells in the presence of androgen and after 8, 24, 48, 72 hours of androgen deprivation, relative to POLR2F; (D) Representative smiFISH images of PROCA11 in cells growing in the presence and absence of hormone (DHT and NE-72h, respectively), dashed lines represent the outline of the nucleus; (E) Violin plot representation of the number of PROCA11 transcripts per cell in the DHT (N=85) and NE-72h conditions (N=86) (p-value <  $4.5 \times 10^{-11}$ , Student t.test), and (F) Percentage of the PROCA11 nuclear foci per cell quantified by smiFISH and ImageJ. Asterisks indicate p-values range from 0.05 (\*) to 0.0001 and below (\*\*\*\*); (G) RTqPCR quantification of PROCA11 in cytoplasm, nucleoplasm and chromatin fractions of cells growing in the absence of androgen for 72 hours.

To sum up, androgen deprivation of adenocarcinoma cells induces drastic transcriptomic changes with the most significant increase in levels of a novel lncRNA PROCA11. The *PROCA11* gene is composed of 7 exons that can be spliced in three variants, all showing mostly nuclear residence but distinct distribution between the nucleus and the cytoplasm. This can determine different fates and activities of PROCA11 isoforms in cells. Androgen deprivation leads to a release of AR from the *PROCA11* promoter and burst of its transcription very early in the onset of the NE phenotype. Therefore, we speculated that PROCA11 may contribute to acquisition of castration resistance as part of CRPC and NEPC transcriptomes.

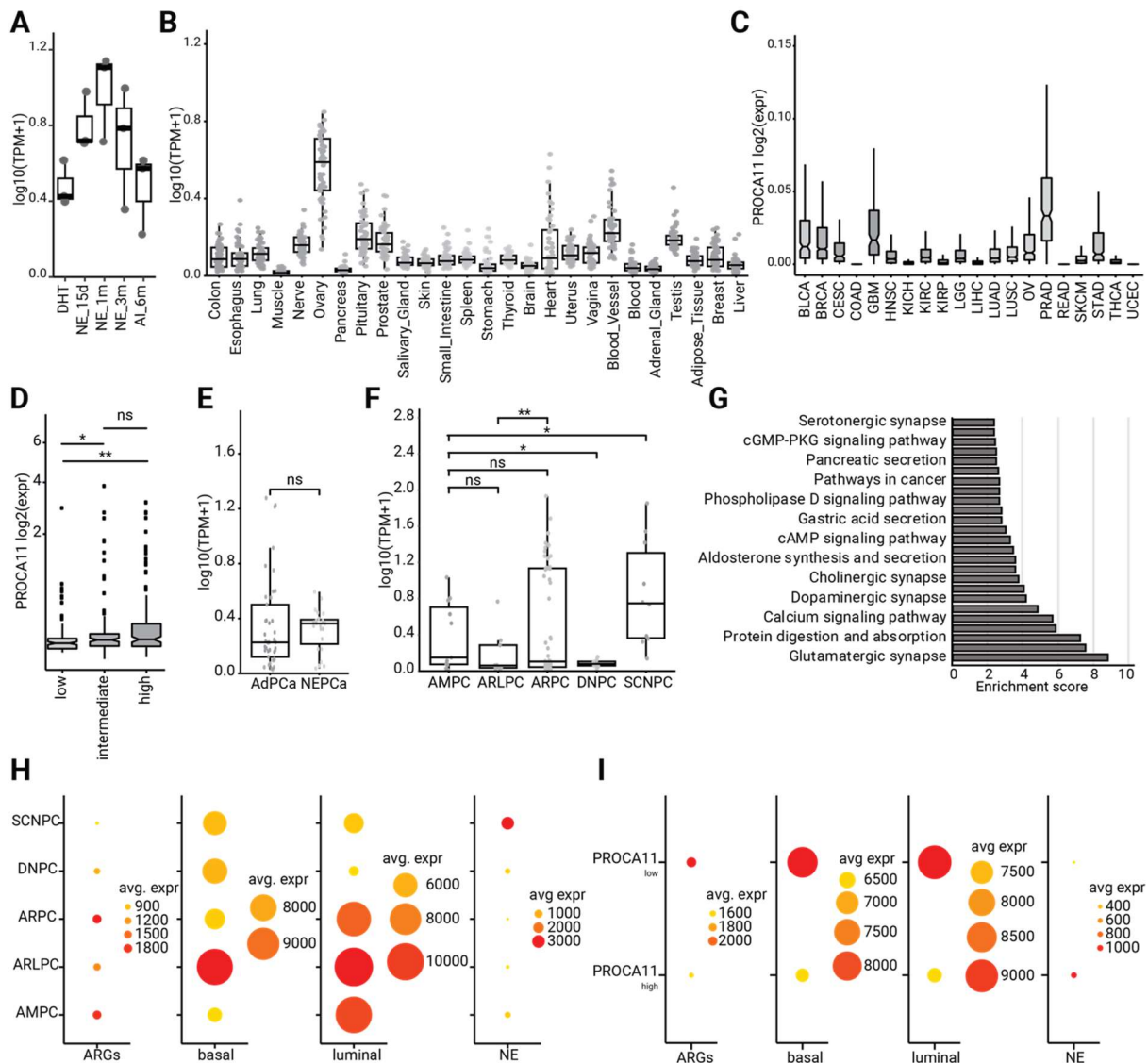
### PROCA11 expression is elevated in ADT-treated adenocarcinoma, CRPC and NEPC tumors.

To get insights into clinical relevance of the newly discovered lncRNA, we explored the expression of PROCA11 in numerous publicly available clinical datasets and compared it to our LNCaP system. First, the Genotype-Tissue Expression (GTEx) query revealed rather low PROCA11 expression in normal tissues (more than 100 times less) than in our primary adenocarcinoma cells with an exception of the ovary where it reached basal expression of the AD-cells growing in the presence of androgen (Carithers *et al*, 2015) (Figure 3A-B).

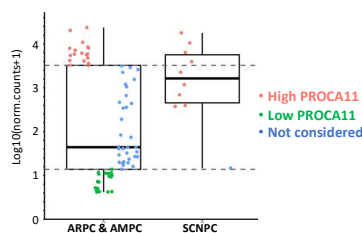
We next evaluated PROCA11 levels in cancer tissues across The Cancer Genome Atlas (TCGA) cohorts. As expected, it was markedly elevated in prostate adenocarcinoma (PRAD), but also notably high in glioblastoma multiforme (GBM), and moderately abundant in bladder urothelial carcinoma (BLCA) and breast invasive carcinoma (BRCA) (Figure 3C). Significantly, the higher lncRNA abundance in intermediate- and high-risk prognosis specimens of TCGA-PRAD than in low-risk tumors reinforced our hypothesis of PROCA11 association with advanced malignancy (Figure 3D).

Finally, PROCA11 expression was interrogated in metastatic CRPC with 41 tumors classified as castration-resistant adenocarcinoma with still active AR signaling (AdPC) and 23 tumors of neuroendocrine prostate cancer (NEPC) with inactive AR (Beltran *et al*, 2019), (Beltran *et al*, 2011). Remarkably, PROCA11 abundance was comparable in both subgroups ( $p$ -value < 0.05) (Figure 3E). We decided to explore more deeply a relationship between PROCA11, AR and NE genes signatures in another clinical study of post-ADT metastatic CRPCs subclassified into tumors with high expression of AR (ARPC, AR<sup>+</sup>/NE<sup>-</sup>), with low expression of AR (ARLPC, AR<sup>low</sup>/NE<sup>-</sup>), of small cell or neuroendocrine histology with expressed NE genes but lacking AR (SCNPC, AR<sup>-</sup>/NE<sup>+</sup>), double positive amphicrine tumors (AMPC, AR<sup>+</sup>/NE<sup>+</sup>) and, finally, double negative tumors lacking both AR and NE genes expression (DNPC, AR<sup>-</sup>/NE<sup>-</sup>) (Labrecque *et al*, 2019). Remarkably, PROCA11 expression was highest in SCNPC, but still detectable in all tumor subtypes, though surprisingly low in ARLPC and DNPC. This observation confirmed that PROCA11 is expressed in AR-positive cells, but also requires other factors or stimuli provided by NE cells. Although, it remains difficult to conclude if elevated PROCA11 expression is a consequence or a cause of the onset of the NE phenotype. Moreover, our attention was drawn to the observation of a bimodal distribution of the lncRNA in ARPC and AMPC tumors with still active AR signaling reaching the mean expression levels in AR-negative SCNPC (Figure 3F). Hence, we undertook a naïve search for a transcriptomic pattern discriminating the best PROCA11 high (above 75<sup>th</sup> percentile) and low (below 25<sup>th</sup> percentile) tumors of ARPC and AMPC subtypes together with the SCNPC tumors highly expressing the lncRNA (Supplementary Figure 2). Differential expression (FC above 2,  $p$ -value below 0.05) and GO term (FDR below 0.05) analyses across these specimens revealed, among others, up-regulation of genes, whose activities can be associated with proliferation and neurogenesis (cAMP and calcium signaling pathways, synapse) and with secretory pathways typical of luminal cells (Figure 3G). Furthermore, in addition to PROCA11 the top three protein-coding genes most discriminative between high and low PROCA11 tumors were neuron-specific *CNTNAP5* and *NRXN3*, both part of the neurexin family of genes with functions in cell adhesion and intercellular communication in the nervous system, and *MAP2* encoding a protein involved in microtubule stabilization during neurogenesis. Comparison of expression patterns for AR-targeted genes, basal, luminal and NE signatures clearly show that elevated PROCA11 levels are associated with higher expression of luminal genes in AR-positive cells and NE genes in SCNPC, and low PROCA11 levels are associated with higher basal component of AR- and NE-negative DNPC subtype (Figure 3H-I). Finally, AR-positive cells with low PROCA11 expression are characterized by mixed luminal-basal pattern (Figure 3I).

Together, these observations demonstrated that the high PROCA11 expression coincides with the expansion of the luminal signature and of the NE phenotype in CRPC tumors.



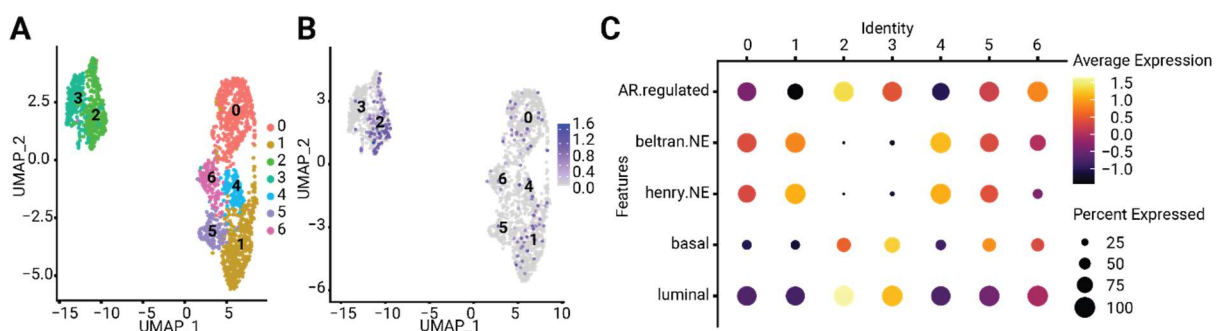
**Figure 3.** PROCA11 expression patterns in normal and tumor tissues suggest strong association with CRPC and NEPC onset: PROCA11 expression quantification in the dynamic LNCaP system of androgen deprivation (A), and across the GTEx collection of normal tissues (B) ; TCGA cohorts (C); TCGA-PRAD depending on tumor risk prognosis (D); AdPC and NEPC tumors from (Beltran *et al*, 2019) (E); and, finally, CRPC tumors from (Labrecque *et al*, 2019) (F); (G) KEGG pathways associated with up-regulated genes in tumors of AMPC, ARPC and SCNPC subtypes with high PROCA11 levels; (H-I) Dot plot of mean expression of genes constituting AR-positive targets - ARGs (Zhang *et al*, 2018), basal, luminal and NE signatures, retrieved from (Henry *et al*, 2018), in CRPC tumors from (Labrecque *et al*, 2019) and only AMPC and ARPC subtypes with high and low PROCA11 levels.



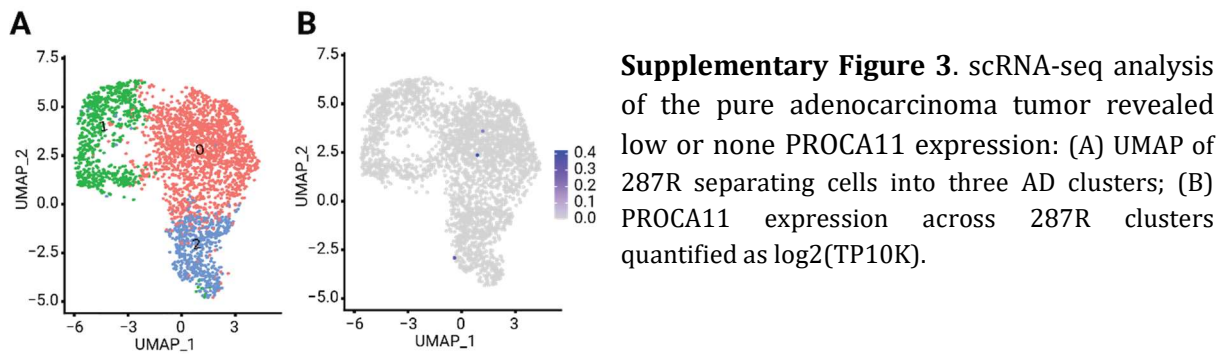
**Supplementary Figure 2.** Assignment of PROCA11 expression levels: as high in SNPC (n=8), but also ARPC and AMPC subtypes (n=19) with lncRNA levels above 75<sup>th</sup> percentile) (red) and as low in ARPC and AMPC subtypes (n=18, with lncRNA levels below 25<sup>th</sup> percentile) (green). Specimens marked in red and green were used for differential expression analysis.

## PROCA11 can be expressed in adenocarcinoma and NE cells of an ADT-naïve tumor.

PROCA11 expression in tumors with still functional AR signaling suggested that AR activity is not the only prerequisite governing lncRNA expression in CRPC tumors. High heterogeneity of tumors treated by ADT also led us to assume that the bulk RNA-sequencing misleadingly represents transcriptomes of distinct cell populations in the CRPC context. Hence, we decided to assess PROCA11 expression in two single-cell (sc)RNA-sequencing datasets from the MURAL collection of ADT-naïve patient-derived xenografts (PDXs) (Risbridger *et al*, 2021). One specimen recapitulated a mixed pathology with AR- and NE-positive cells (224R, AR<sup>+</sup>/NE<sup>+</sup>) and the other corresponded to a homogenous adenocarcinoma with active AR signaling and negative for NE genes (287R, AR<sup>+</sup>/NE<sup>-</sup>). A uniform manifold approximation and projection (UMAP) analysis separated 224R into two adenocarcinoma (AD) clusters and five NE clusters, and 287R into three adenocarcinoma clusters, comparably with the literature (Risbridger *et al*, 2021) (Figure 4A; Supplementary Figure 3B). Remarkably, PROCA11 was detectable only in the 224R mixed tumor, specifically, at high levels in one of the AD clusters, cluster 2, and parsimoniously in cells of all five NE clusters (Figure 4A). The 287R tumor showed low or any presence of the lncRNA (Supplementary Figure 3B). Hence, we concluded that PROCA11 is expressed in ADT-naïve tumors, as expected, in NE cells, but also in AD cells with still active AR signaling within a microenvironment created by NE cells. Further analysis of gene expression patterning across all clusters of the 224R tumor confirmed that PROCA11 is enriched in adenocarcinoma cells of more pronounced luminal cell type and lower basal signature, but also in cells with marked NE identity (Figure 4C). Gene ontology analysis of significant differentially expressed genes in the clusters 2 comparing to the cluster 3 revealed, in cells enriched in PROCA11 levels, notable down-regulation of translation (30 genes of RPL and RPS family, False Discovery Rate, FDR=6.45x10<sup>-31</sup>), and a tendency to down-regulation of the WNT signaling pathway (TIAM1, GPC3/6), pro-apoptotic, and cell cycle genes (*CDKN2A/p21*, *RACK1*). We speculated that AD-cells expressing PROCA11 have predominant luminal phenotype, enduring a particular low-proliferative state avoiding cell cycle arrest.

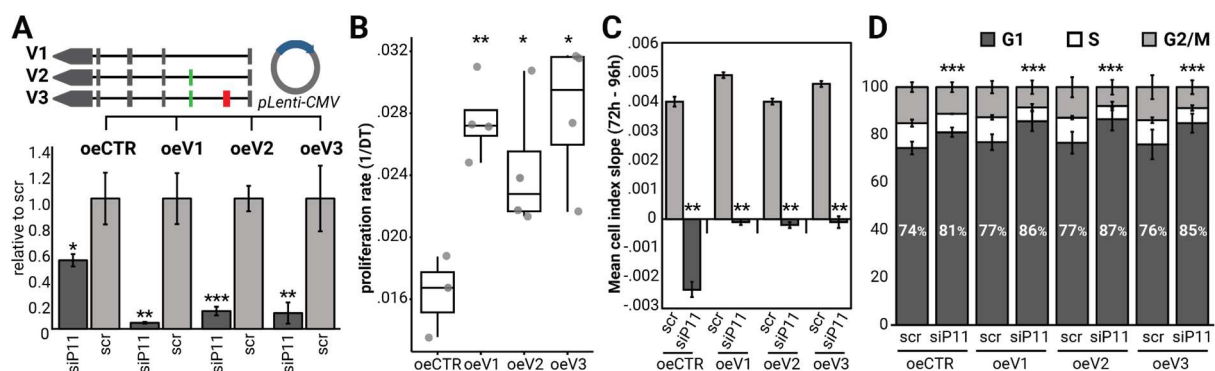


**Figure 4.** PROCA11 is expressed in the ADT-naïve tumor of the mixed adenocarcinoma and NE phenotype: UMAP (A - B) representation showing five NE and two AD clusters (A) and expression levels of PROCA11 (in log<sub>2</sub>(TP10K) based on scRNA sequencing (B)); (C) Dot plot comparing expression of NE, basal and luminal gene signatures from (Henry *et al*, 2018) and (Beltran *et al*, 2016) across the AD and NE clusters for 224R.



### PROCA11 regulates cell proliferation and cell cycle.

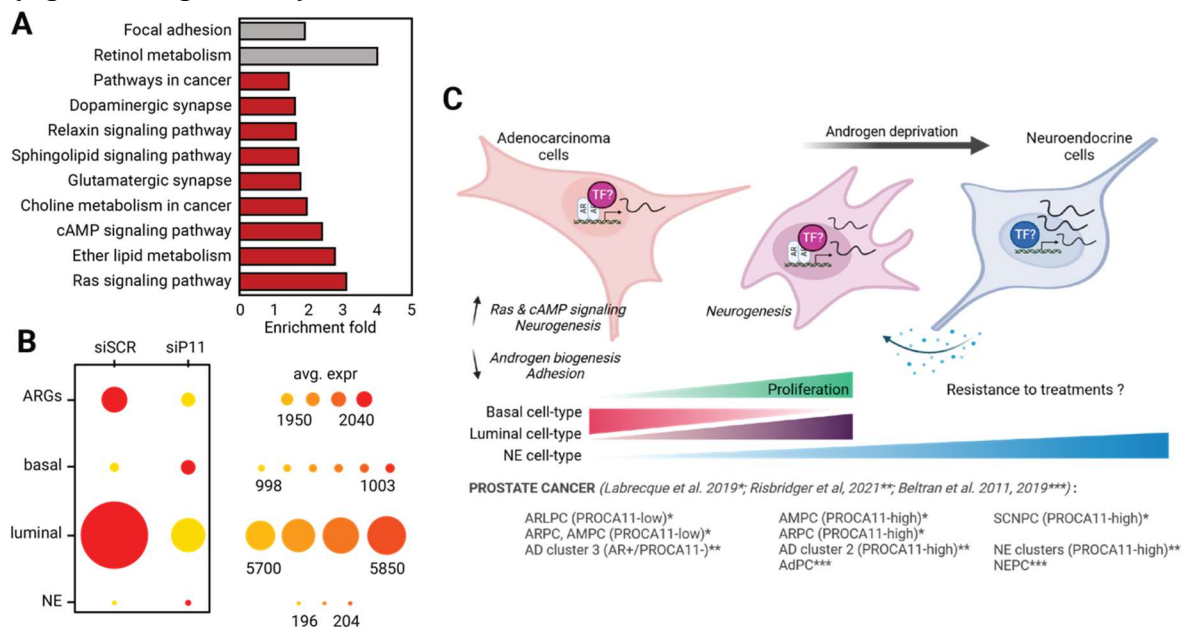
Since in our model the increase of PROCA11 preceded neuroendocrine transdifferentiation and growth arrest, we hypothesized that it might have an effect on proliferation of cells. We thus performed gain- and loss-of-function studies to determine if alterations in lncRNA expression change cancer-related phenotypes. First, we sub-cloned the three major lncRNA variants into a lentiviral construct under the control of the constitutive CMV promoter and transduced them into LNCaP (oeP11) to overexpress each of three variants (oeV1, oeV2 and oeV3, respectively). To decrease PROCA11 levels we used an siPOOL strategy transfecting LNCaP cells growing in the presence or the absence of androgen with a mix of thirty siRNAs targeting exons common to all PROCA11 variants (siP11) or non-targeting controls (siSCR) (Figure 5A). PROCA11 depletion by siP11 was efficient achieving 80-90% of decrease in cells over-expressing PROCA11 variants though less robust (no more than 50%) in physiological conditions (Figure 5A). To follow cell proliferation, we used an xCELLigence real-time cell assay (RTCA) based on continuous measurements of electrical impedance. AD-cells and oeV1/V2/V3 cells overexpressing PROCA11 were seeded on plates, transfected or not with siRNAs and monitored during 260 hours for proliferation. Stable and constitutive PROCA11 overexpression resulted in faster proliferation rates for all three lncRNA isoforms (Figure 5B). And reverse, transient PROCA11 depletion in AD-cells and oeV1/V2/V3-cells reduced proliferation (Figure 5C). Proliferation depends on progression of a population of cells through the cell cycle. Hence, we assessed it in cells using flow cytometry and propidium iodide staining of DNA. PROCA11 depletion resulted in up to 10% increase in a proportion of cells in G1 over S and G2/M phases (Figure 5D). Collectively, these results suggested that PROCA11 contributes to proliferation in the presence of androgen and may play a role in cell cycle progression. Although, all three isoforms seem to contribute similarly to lncRNA-induced phenotype changes.



levels in AD-cells and cells over-expressing PROCA11 (oeV1, oeV2, oeV3) transfected with siP11 relative to siSCR; (B) Proliferation rate (doubling time,  $DT^{-1}$ ) of oeCTR, oeV1, oeV2, oeV3 cells measured from 0 to 96 post-seeding ; (C) Cell index slope assessed for the exponentially growing cells transfected with siSCR or siP11 from 72 to 96 hours post-transfection; (D) Proportion of cells in G1, S and G2/M phases of the cell cycle. Asterisks represent the p-value range from 0.05 (\*) to 0.001 and below (\*\*\*) (Student t.test).

### PROCA11 modulates metabolic and neurogenic activities in adenocarcinoma cells.

In light of our findings that PROCA11 is expressed in AR-positive cells ubiquitously present in CRPC and that it regulates cell proliferation, we further investigated transcriptomic changes induced by the depletion of the lncRNA in AD-cells. Differential expression analysis of protein-coding genes identified 90 up- and 120 down-regulated genes ( $|FC|$  above 1.5, adj.p-value below 0.05). Strikingly, genes with increased expression were mostly part of Ras signaling, neurogenic activities, as glutamatergic and dopaminergic synapse, relaxin, cAMP and sphingolipid signaling pathways (Figure 6A). Notably, among up-regulated genes were several with important activities in neuron cell homeostasis and plasticity, as the MAPK10 signaling kinase, several genes encoding PLA2 enzymes of the fatty acid metabolism, or the DDC enzyme catalyzing the production of neural transmitters. On the other hand, down-regulated genes were associated with retinol metabolism and focal adhesion (Figure 6A). Among them, several genes belonged to the UGT2B family of UDP glucuronosyltransferases and to the cytochrome P450 family of enzymes, involved in the metabolism of different steroid hormones including androgens (Chouinard *et al*, 2007), (Mitra & Goodman, 2015). In addition, depletion of PROCA11 also led to an overall decline of the mean expression of the luminal cell-type signature and expansion of the basal signature (Figure 6B), as it was also determined by the scRNA-sequencing of the mixed AD/NE tumor in the AD cluster 3 with low or undetectable PROCA11 levels, but also in CRPC tumors with low PROCA11 (Figure 4C, Figure 3H-I).



**Figure 6.** Alterations in PROCA11 expression change metabolism, adhesion and neurogenesis related pathways: (A) KEGG pathways enriched for up- and down-regulated genes (n=90 and 120, respectively) in response to PROCA11 depletion in AD-cells. Only genes with the expression  $|FC|$  above 1.5 were considered in GO term analysis by DAVID. (B) Dot plot comparing expression of NE and luminal gene signatures from (Henry *et al*, 2018) in AD-cells treated with siSCR or siP11; (C) Model of PROCA11 activity in the onset of CRPC and NEPC (created with BioRender.com).



In conclusion, our results strongly suggest that in cells growing in the presence of androgen PROCA11 contributes to the maintenance of the AR signaling to preserve a proliferative potential, modulates cellular plasticity switch from basal to more pronounced luminal cell type and potentiates cells to acquisition of NE properties.

## Discussion

Emerging examples suggest that lncRNAs intervene in fine tune regulation of many cellular processes modulating cell identity and fate (Chouinard *et al*, 2007), (Mattick *et al*, 2023). In cancer, lncRNAs are proposed to influence tumor progression and response to therapies (Carlevaro-Fita *et al*, 2020), (Vancura *et al*, 2021). In this work we discovered a novel lncRNA PROCA11 with a possible role in the onset of castration-resistant and neuroendocrine prostate cancer.

Transcriptome profiling of the dynamic *in vitro* system of the prostate adenocarcinoma revealed PROCA11 as the most activated in response to androgen deprivation. Importantly, PROCA11 levels are elevated in tumors of high Gleason grades, ADT-treated adenocarcinoma and NEPC tumors. Therapies targeting AR signaling, such as ADT, affect the plasticity of PCa cells by inhibiting the capacity of AR to activate transcription of proliferation and pro-survival genes. On the other hand, ADT results in a release of AR and activation of genes involved in stem-cell fate, epithelial-to-mesenchymal transition or neuroendocrine transdifferentiation (Beltran *et al*, 2016), (Nouri *et al*, 2017). Similarly, androgen ablation in the adenocarcinoma cell line induces, otherwise low, transcription of *PROCA11*. It coincides with the AR release from the lncRNA promoter and persists in growth arrested cells undergoing the neuroendocrine transdifferentiation. PROCA11 expression is also stimulated by the depletion of AR in the presence of androgen. Together, these findings strongly suggest either a negative regulation of lncRNA transcription by AR or through two distinct mechanisms: AR-mediated basal transcription in the presence of hormone, and strong activation by another transcription factor (TF) upon androgen withdrawal. *In silico* search for TF binding sites identified response elements of the basal TBP subunit of TFIID, of the AR, but also of the AP-1 complex members (JUN and FOS), several TFs (FOS, ESR1 and USF2), some other ligand-dependent TFs (GR, RAR/RXR), and differentiation TFs (POU2F2, LEF1, FOXA1), all of them are up-regulated in response to androgen deprivation. Interestingly, elevated ER $\alpha$  expression and downstream activation of its targets (TMPRSS2-ERG, PS2 or the NEAT1 lncRNA) were reported in AR-negative cells and PCa tumors (Setlur *et al*, 2008).

PROCA11 is not or very lowly abundant in almost all normal tissues with the exception of the ovary, but increases in advanced PCa tumors subjected to ADT, and specifically in tumors with NE features. ADT induces very heterogeneous changes in tumors, containing cells with still active AR signaling but also cells with inactive AR, cells acquiring stem-cell like or neuroendocrine features (Labrecque *et al*, 2019). In all studied clinical cases elevated PROCA11 levels were associated with ADT and co-expression of NE signature. However, scRNA-sequencing also revealed PROCA11 presence in the ADT-naïve tumor of the mixed adenocarcinoma and neuroendocrine phenotypes, but not in the pure homogeneous adenocarcinoma. Notably, it was expressed in AR-positive AD-cells presenting features of low or non-proliferative cells. In this context, PROCA11 may be part of a regulatory circuit preventing cell cycle arrest and apoptosis. One can also speculate that a particular NE microenvironment contributes to PROCA11 expression. NE cells secrete a variety of peptides and proteins with endocrine and paracrine effects, modulating the secretory activity of luminal cells to regulate prostatic growth and

function (reviewed in Arman & Nelson, 2022). Therefore, in heterogeneous mixed tumors, NE cells' secretome can activate a specific response, remodeling the transcriptome of adjacent adenocarcinoma cells stimulating PROCA11 expression and endowing them with the neuroendocrine properties and preserving their proliferative potential. Single-cell resolution studies in cells and tissues will shed light on the intricate relationship between PROCA11, the AR signaling and the onset of CRPC and NEPC phenotypes.

Depletion of PROCA11 in androgen-dependent cells leads to decreased cell proliferation and perturbed cell cycle with more cells in G0/G1 phase. Ectopic overexpression of PROCA11, in the contrast, promotes cell proliferation. Functional association analysis with GO analysis demonstrated that down-regulated genes are enriched for androgen biogenesis, as though up-regulated genes are involved in Ras and cAMP pathways. Activation of the Ras signaling in ADT treated prostate cancer was reported to reduce the androgen requirement of LNCaP cells for growth (Bakin *et al*, 2003). In a similar way, activation of the cAMP pathway was showed to promote AR translocation into the nucleus in conditions of low androgen levels, also stimulating AR signaling and survival (Dagar *et al*, 2019). Hence, activation of these two pathways in response to PROCA11 depletion converges to sensitize the AR activity to subphysiological levels of androgen. In the brain, cAMP function is tightly linked to synapse formation and neuron plasticity (reviewed in Shahoha *et al*, 2022). In PROCA11-depleted cells the cAMP pathway activation is concurrent with an increase in expression of genes involved in synaptic activities. These findings strongly point to the role of PROCA11 in regulation of cell plasticity enabling survival and growth but at the same time the emergence of the NE phenotype in conditions of perturbed androgen supply. This regulatory mechanism implies an intricate cross-talk between AR and PROCA11 and possible feedback loops with metabolic and neurogenic signaling pathways.

In the context of still functional AR signaling, elevated PROCA11 levels in our dynamic system, CRPC tumors and ADT-naïve tumor of the mixed AD/NE phenotype was associated with increased expression of luminal cell-type genes in detriment of basal cell-type genes. According to lineage tracing studies, basal cells have the ability to differentiate into luminal cells (Choi *et al*, 2012). Mixed basal-luminal (intermediate) population of cells resists to pharmacological castration and was suggested to given rise to CRPC. We speculate that in tumors PROCA11 may be involved in regulation of basal-luminal balance, contributing to the emergence of persister cells maintaining their proliferative potential and sensitized to neuroendocrine trans-differentiation. Therefore, ADT may induce the expression of PROCA11, first, as a compensatory response to counteract the growth arrest, and then enable to modulate cellular plasticity in favor of basal-to-luminal transformation and emergence of the NE phenotype.

The precise molecular mechanisms triggered by PROCA11 in the androgen-dependent and independent contexts still stay to decipher. So far, *in silico* predictions of the coding potential and studies of cell phenotypes induced by expression of different PROCA11 isoforms did not support their distinct fate and activity in cells. However, in light of recent findings on bifunctional lncRNAs, we cannot exclude that the shortest V1 and V2 isoforms, more enriched in the cytoplasm than V3, are translated, giving rise to a peptide with potential regulatory functions.

## Material and Methods

### Cell lines and culture

The LNCaP cell line was purchased from ATCC (CRL-1740), cultured at 37°C in a humid 5% CO<sub>2</sub> atmosphere in either the RPMI 1640+GlutaMAX medium (Life Technologies) supplemented with 10% fetal bovine serum and 2 nM DHT (AD-cells, or +DHT) or the phenol red-free RPMI 1664+GlutaMAX medium supplemented with 10% dextran charcoal-stripped fetal bovine serum (NE- and AI-cells). HEK293T was purchased from ATCC (CRL-3216), cultures in DMEM + GlutaMAX (Life Technologies) supplemented with 10% fetal bovine serum. Cells were systematically tested and free of mycoplasma.

### Plasmids

The PROCA11 cDNA was obtained by reverse transcription and PCR with primers complementary to 5' and 3' extremities of the gene, V1, V2 and V3 amplicons were resolved in the 1% agarose gel and purified using the gel purification kit (Qiagen). Each cDNA was cloned into pDONR201 (Invitrogen) and further sub-cloned into pLenti CMV Blast DEST (706-1) (Addgene, #17451) (Campeau *et al*, 2009) using the Gateway system (Thermo Fisher Scientific). Constructs were validated by Sanger sequencing.

### Transfections and lentiviral transduction

Transfections were performed at 50-70% of cell confluency using the Jetprime® transfection kit (Polyplus) following the manufacturer's protocol: (i) LNCAP with siPOOL (siTOOLS Biotech GmbH) targeting PROCA11 (siP11), AR (siAR) or non-targeting scramble (siSCR) at a final concentration of 30nM. Experiments were performed 48 hours post-transfection; (ii) HEK293T with 1.3 µg of psPAX2 (Addgene, #12260), 0.8 µg of pVSVG (Addgene, #36399) and 0.8 µg of the lentiviral plasmid hosting the cDNA of GFP lacking the first ATG (CTR) or PROCA11 variants (V1, V2, V3). 48 hours post-transfection, viral supernatant was collected, filtered and added to cells at 70% of confluency at 0.5 MOI for 24 hours. Transduced cells were subcultured in the medium supplemented with blasticidin at 6 µg/ml for four passages prior to use.

### Subcellular fractionation

The protocol was adapted from (Gagnon *et al*, 2014) for 10<sup>7</sup> cells. All steps were performed on ice with ice-cold buffers supplemented with 20 U/µl SUPERase-IN (Thermo Fisher Scientific) for RNA extraction and 0.1 mM AEBSF (SIGMA) for protein extraction. For nascent transcription experiments,  $\alpha$ -amanitin was added in the HLB buffer at 25µM concentration. RNA extraction from all fractions was performed using Qiazol at 65°C for 5 minutes with regular mixing and then purified with miRNeasy kit (Qiagen) according to the manufacturer's instructions. For protein extractions, the chromatin fraction was sonicated for 4 min (30s-on/30s-off, "high power") on the Bioruptor Plus (Diagenode). Proteins' content was quantified with the Thermo Fisher Scientific™ BCA Protein Assay Kit (Thermo Fisher Scientific).

### RNA extraction and reverse transcription

Total or AR-immunoprecipitated RNA extraction was performed directly from cell cultures with miRNeasy kit according to the manufacture' instructions including the DNase treatment step (Qiagen). Reverse Transcription (RT) was performed on 500 ng of RNA with random primers and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific).

### Quantitative PCR

cDNA was diluted 10-50 times and quantified by real-time PCR with LightCycler® 480 SYBR Green I Master (SIGMA). The *RPL11*, *POLR2F* or *ACTB* genes were used as reference genes to

quantify a relative abundance of each cDNA. Error bars represent standard deviations in relative quantity of cDNA prepared from at least three independent experiments.

### **Cell cycle analysis**

In total, 200000 cells were recovered after trypsinization, washed twice in PBS, resuspended in 500 $\mu$ l of ice-cold 70% EtOH and incubated at 4°C for 30 minutes. Subsequently, cell pellets were treated with 50 $\mu$ l of RNase A solution (100 $\mu$ g/mL) for 5 minutes at room temperature and stained in 300 $\mu$ l of 50 $\mu$ g/mL propidium iodide solution for the next 30 minutes. Cells were processed using the NovoCyte flow cytometer (Agilent). Data was acquired for 10000 single cells per condition and analyzed by the FlowJo software (v10.9.0).

### **Proliferation**

Cells were seeded (8000 cells/well) in a 96-well RTCA E-plate (Agilent) in 4 replicates per condition. Following a short spin at 130g for 1 minute, cells were transfected with siSCR or siP11 as described above. Electrical impedance was measured for 260 intervals of 1 hour using the RTCA xCELLigence device (Agilent). Cell index slope and doubling time were retrieved from the software and used for statistical analysis in R.

### **RNA-seq analysis**

Read alignment files were used as input for running the Scallop v0.10.5 transcriptome assembler with default options (Shao & Kingsford, 2017). Assemblies were merged and mapped to the reference annotation Gencode Release 32 (GRCh38.p13) by the Cuffmerge v1.0.0 tool (Trapnell *et al*, 2010). All non-annotated transcripts with class code “u” (intergenic, n=4120 transcripts, n=2582 genes), “x” (antisense, n=2837 transcripts, n=1629 genes) and code “j” (sense exonic overlap, n=35 transcripts) restricted only to noncoding genes were retrieved and added to GRCh38.p13 to make a new custom transcriptome assembly. Reads were mapped using Tophat v2.0.4 (max mismatches allowed = 3) (Kim *et al*, 2013); (Trapnell *et al*, 2009). The uniquely mapped reads were counted at the gene level using featureCounts v2.0.0 (Liao *et al*, 2014) and the custom assembly build and, then, processed by DESeq2 v1.26.0 (Love *et al*, 2014) using the DESeq R package (Anders & Huber, 2010). Luminal, basal, NE gene signatures were retrieved from (Henry *et al*, 2018), ARGs were retrieved from (Zhang *et al*, 2018).

### **Correlation expression matrix and gene ontology**

A correlation matrix was built between each lncRNA and the totality of differentially expressed PCGs (n=6982) obtained from all the time points of hormonal deprivation (15 days, 1 month, 3 months, 6 months) using the pcor() in R. Highly correlated PCGs (|pearson r| >= 0.99) were selected for the gene ontology analysis with the R package clusterProfiler v3.14.3 (fun=enrichGO, ont=“BP”, pvalueCutoff =0.2). PROCA lncRNAs (n=15) make part of the functional group ([*nerv / neur / axon / synap*]).

### **Public bulk and single-cell RNA-seq datasets query**

Read counts from TCGA were retrieved by TANRIC (Li *et al*, 2015). Tumor classification as low-, intermediate or high-risk group was performed according to Gleason and TMN scores as previously described (Pinskaya *et al*, 2019).

Expression in normal tissues was obtained from the GTEx Portal via the SRA cloud data delivery service, by using the dbGaP eRA credentials for accession number phs000424.v9.p2, and the google cloud platform for storage on 12/15/2022 (<https://gtexportal.org/home/>) (Carithers *et al*, 2015). CRPC RNA-seq datasets were accessed through the NCBI GEO data repository under the number GSE126078 (Labrecque *et al*, 2019), and through dbGaP under the number phs001666.v1.p1 (Beltran *et al*, 2019). ScRNA-seq datasets of PDX 224R and 287R MURAL

collection were downloaded from the Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/sra/>) under the BioProject accession PRJNA675382 and analyzed as described in (Risbridger *et al*, 2021).

### **smiFISH**

Probes design and procedure were carried out following the instructions of (Tsanov *et al*, 2016).

### **Microscopy image analysis**

Image analysis was carried using the ImageJ2 Fiji package (Schindelin *et al*, 2012). Stacks, spaced out by 0.6µm, were merged using *maximum intensity Z-projection*. Two regions of interest per cell defined total and nuclear areas. PROCA11 foci were counted using *find maxima* and the threshold = 80. Cytoplasmic counts were obtained by subtracting total with nuclear counts.

### **CUT & RUN**

The experiment was performed in two replicates following the protocol from Henikoff lab (Skene *et al*, 2018) with modifications described in (Jarroux *et al*, 2021). DNA fragments were purified with NEB Monarch PCR & DNA purification kit following the protocol enrichment for short DNA fragments and was quantified by Qubit HS DNA kit.

### **CUT & RUN libraries preparation and sequencing**

CUT & RUN libraries were prepared using the TruSeq ChIP-Sample Prep Kit-Set A and PCR Box (Illumina) from 5 ng of DNA with 10 PCR cycles for amplification following the manufacturer's procedures. Concentration and quality were assessed by Qubit with dsDNA HS Assay Kit (Thermo Fisher Scientific) and High Sensitivity DNA Analysis Chip Kit (Agilent Technologies). Libraries were sequenced on a NovaSeq – S1 device in a pair-end 100 bp read length mode.

### **CUT & RUN analysis**

Reads were first aligned to human genome version GRCh38 using bowtie (version 2.4.1) (Langmead & Salzberg, 2012), with options -I 10 -X 700 --no-mixed. Reads with MAPQ value  $\geq 30$  were kept for further analysis. To equilibrate datasets, the larger samples were randomly down-sampled. For AR, peaks were called relative to the IgG control using MACS2 version 2.2.6 (ref) and options: -f BAMPE -g hs -B --keep-dup all. For further analysis, common peaks between conditions were also merged to peak regions. The merged peaks were assigned to closest genes and the shortest distance between each peak and the closest transcription start site (TSS) coordinates were calculated using the Gencode v32 annotation. The AR binding site within the PROCA11 region was inferred using PROMO (Messeguer *et al*, 2002).

### **Gene features analysis**

Gene ontology (GO) biological process, KEGG pathways and cellular compartment terms enrichment analyses were performed using DAVID (Huang *et al*, 2008). The gene enrichment score was calculated as  $-\log_{10}(P\text{-value})$ .

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## Author Contributions

Project administration, funding acquisition, conceptualization, cosupervision and writing, A.M.; conceptualization, cosupervision and writing, Ma.P.; draft revisions, Ma.P., R.C., A.M.; investigation R.C., Ma.P., V. F., Mi.P., G.I.R.; formal analysis, R.C., M.G., U.S.

**The authors declare no conflict of interest.**

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## 1.1. Molecular characterization of PROCA11 function

We showed that depletion of both physiological and overexpressed PROCA11 inhibits cell proliferation. LncRNAs typically exert their influence primarily through RNA-centric processes, involving RNA-RNA and RNA-protein interactions, or alternatively, by being translated into small peptides. To fully characterize the functional role of lncRNA PROCA11, we sought to explore both potential mechanisms of action and we began by investigating its coding potential.

### 1.1.1. Exploring a coding potential of PROCA11.

In an initial attempt to ascertain whether the lncRNA PROCA11 encodes peptides or small proteins, we utilized CPAT (v3.04) to predict the coding potential of its primary sequence. CPAT analysis revealed that none of the PROCA11 isoforms possess coding potential (Table 1).

**Table 1. CPAT coding potential prediction of PROCA11 isoforms.**

ID	RNA size	ORF size	Ficket Score	Hexamer Score	Coding Probability	Coding Label
V1	2322	264	0.7801	-0.099	0.027	no
V2	2421	264	0.7801	-0.099	0.027	no
V3	2511	261	0.7855	-0.098	0.027	no

However, *in silico* prediction of ORFs, based on ATG and stop codon search, revealed 10 coding sequences, some shared between isoforms and some variant-specific (Table 2).

To investigate a possibility of each lncRNA variant to be translated, we performed sucrose gradient fractionation, RNA isolation from each fraction and quantification of PROCA11 by RT-qPCR. LNCaP cells were grown in the presence of androgen, pretreated with either vehicle (DMSO) or puromycin, which specifically disrupts translating ribosomes, and fractionated following the protocol of Baudin-Baillieu *et al* (Baudin-Baillieu *et al*, 2016).

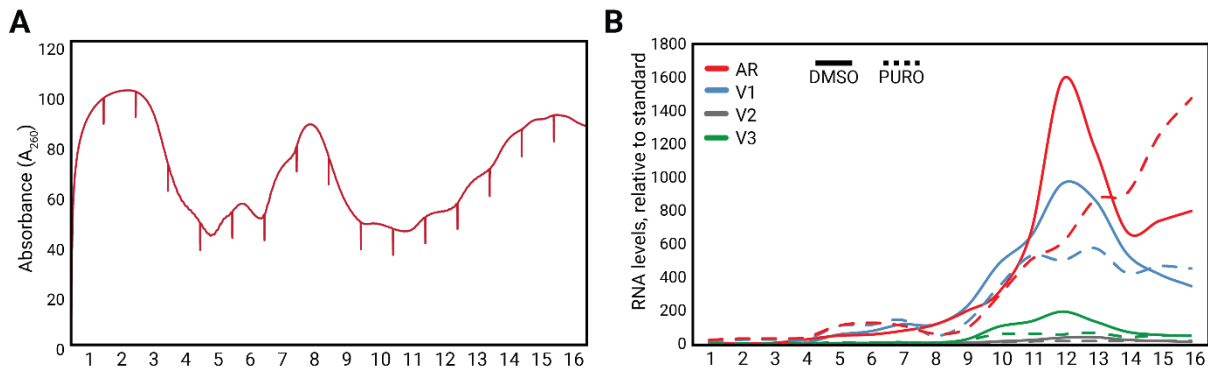
In total, 15 to 16 fractions were collected with lightest fractions (1-4) corresponding to ribonucleoprotein complexes (RNPs), 5-6 to 40S, 7 to 60S, 8-9 to 80S, and finally 10-16 to polysomes (Figure 1A). To discriminate between PROCA11 variants we used primer sets specific to each. AR mRNA was used as a positive control of actively translated RNA in our experimental conditions. First, AR mRNA showed the expected enrichment in the polysome fraction, which was partially disrupted by addition of puromycin (Figure 1B).

**Table 2. ORFs and corresponding peptides predicted in PROCA11 isoforms V1, V2 and V3.**

ID	source	CDS	CDS size (bp)	peptide sequence	peptide size (aa)
ORF1	V1, V2, V3	ATGGAAGCAATAAGAGCAACAAACATTTACACAGTTCTCAGGT CCCTGGACCATGAAAGGCCACATCCAAGGAGCTACCCACGGA GGCCTCTCATTTGCATTTGTCCCCGCTAA	114	MEAI RATNI YTVLRSL DHEGPHPRSYPRRPLI CI CPR	37
ORF2	V1, V2, V3	ATGAAGGGCCACATCCAAGGAGCTACCCAGGAGGCCCTCTCAT TTGCATTTGTCCCCGCTAATGCAGGACAGAGATCATGGAAATTG GGCCCCGACTGGATGA	102	MKGHI QGATPGLSFA FVPANAGQRSWKL GPT G	33
ORF3	V1, V2, V3	ATGGAAGTTGGGCCGACTGGATGAGATTGGGGGTCTTTGGA GTGGAGAAATGTGTTGCCCTGGGGAAGAGAGGTGATGTCTAGG AGACCTTCTCACTCAGGGCAAGCTCTATGA	117	MEVGPDWRFGLVKW RMCLPWGREVMSRRPL HSGQAL	38
ORF4	V1, V2, V3	ATGAGACAGCAGAAAGAAATCAACCTACCAACAGAACTCTGA GAAATGCTCACTTTACTTCAAGCTGCTGCAITTTTGATGTTATG AAACCATAA	96	MROQKEYQPTNRI LRN AHSYFKLLHFDV/MKP	31
ORF5	V1, V2, V3	ATGCAGAGGCAACTCTGGTATTTGTGTGCACTGGCAGCTGATCAT ACCCAGCATGCACGCACTCTCAGGGCAGAGCTGATGCCCCAC ATAATGGGGCATGGTAAGGACTCCCATGTCCTTGAGATAAAAT GA	132	MQRQSGI CVHWQLI I P SMHALSGQSLMPHI MG HGKDSHVLEI K	43
ORF6	V1, V2	ATGCAGGACAGAGATCATGGAAAGTTGGGCCGACTGGATGAGA TTTGGGGGTCTTTGGAAAGTGGAGAAATGTGTTTGGCCCTGGGGAAG AGAGGTGATGCTAG	102	MQDRDHGSWARLDEI W GSLEVENVFALGKRGD V	33
ORF7	V1, V2	ATGTTTTGGTTGTTTCAGTCTTTACAGATGTATCGAAAGGCTGTC CAAAAAATGTCTGACTTCTGTAGTGTGCCTTCGTAGCACTAGAGG GTTCTGCAGCTGTGAAACTGAAAGGCAATGGCAAGAACCCCTCC ATCTTCAAACCACTTTCTGCTAAGGTGTGGCTCTATAAAACCA TATGAGCTGGGCTTCACTGAGACTGAGTTGTTTCAGTAGGATCT TCCGGAAAAACATGTTCCCTCTTACCTGGGTTGTCATTCTGA	264	MFVLFQSLQMYRKAVQ KCLTSVVCLRSTRGFC SCETEGI GKKPSI FKP LSAKVWL YKTTMSVAS LRLSCFSRI FRKNMFP LPGLSF	87
ORF8	V2, V3	ATGAACACTGAAAGTGAAGTGAAGAGACTTTTGCAGTCAITTTT TTGCTGGCTGCAGATGACAGTGACACCCCTGGGAGTTGAGAAAT GCTTGTAAGATGGAAGGAGGCTGTGTCCTGAATGA	123	MNTEVSEKRLLSQFFC WLQMTVTPWELRNACK MEGGCVPE	40
ORF9	V3	ATGCAGGACAGAGATCATGGAAAGTTGGGCCGACTGGATGAGA TTTGGGGGTCTTTGGAAAGTGGAGAAATGTGTTTGGCCCTGGGGAAG AGAGGTGATGCTAG	102	MQDRDHGSWARLDEI W GSLEVENVFALGKRGD V	33
ORF10	V3	ATGTTTTGGTTGTTTCAGTCTTTACAGATGTATCGAAAGGCTGTC CAAAAAATGTCTGACTTCTGTAGTGTGCCTTCGTAGCACTAGAGG GTTCTGCAGCTGTGAAACTGAAAGGCAATGGCAAGAACCCCTCC ATCTTCAAACCACTTTCTGCTAAGGTGTGGCTCTATAAAACCA TATGAGCTGGGCTTCACTGAGACTGAGTTGTTTTCAGTAGGATCT TCCGGAAAAACATGTTCCCTTACCTGGGTTGTCATTCTGA	261	MFVLFQSLQMYRKAVQ KCLTSVVCLRSTRGFC SCETEGI GKKPSI FKP LSAKVWL YKTTMSVAS LRLSCFSRI FRKNMFP LPGLSF	86

Remarkably, only the V1 variant of PROCA11 has a profile comparable to AR mRNA and sensitive to puromycin treatment (Figure 1B). We concluded that V1 is associated with translating ribosomes and may indeed undergo translation. Our subcellular fractionation experiments revealed uneven distribution of PROCA11 isoforms in cells, V1 and V2 being more enriched in the cytoplasm than V3. Intriguingly, V2 did not show association with polysomes. Hence, we hypothesized that the fate of these RNAs in the cytoplasm is different, possibly due to different intrinsic properties of each transcript. To validate our hypothesis of translation, additional experiments are required. A preliminary approach would be to determine whether translation is necessary to display the phenotype observed in PROCA11 by *gain-of-function* experiments, and this can be achieved by inserting a short-hairpin structure at the 5' end of the transcript. It has been shown that the presence of a stable secondary structure close to the 5' end prevents ribosome

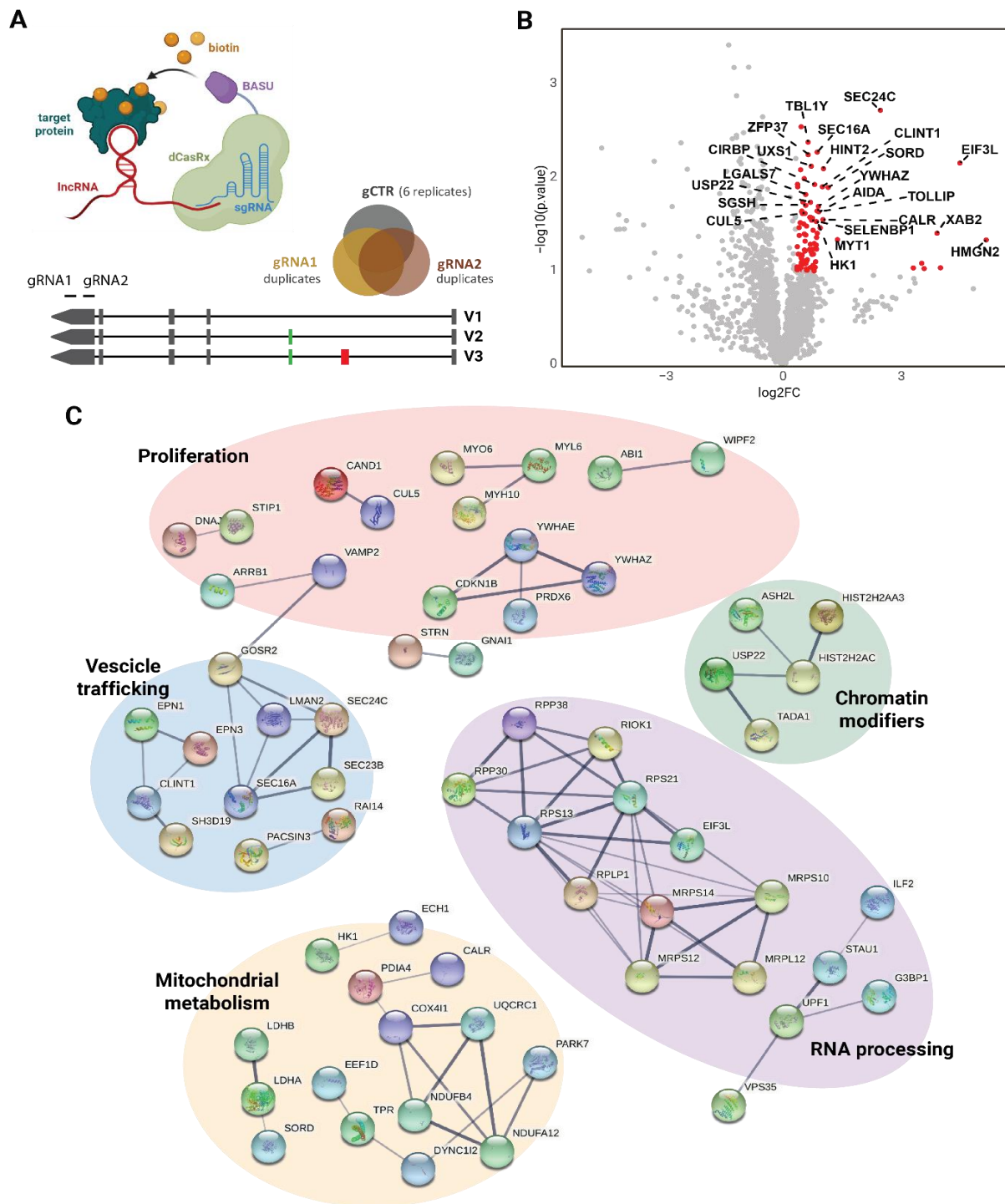
scanning, thus inhibiting translation (Babendure *et al*, 2006). Second, the detection of a peptide product by western blot and immunofluorescence will be crucial and can be facilitated by the ORF fusion to a FLAG-tag. Finally, co-immunoprecipitation of our tagged peptide will enable the identification of protein interactors and give us deeper insights regarding the biological function.



**Figure 1. PROCA11 is associated with translationally active polysomes.** (A) Polysome profiling of LNCaP NE-1m. (B) RT-qPCR of AR and PROCA11 variants in different fractions of LNCaP NE-1m untreated (DMSO) and puromycin treated

### 1.1.2. Identification of protein partners of PROCA11.

While probing the potential peptide-coding capacity of PROCA11, we concurrently pursued the exploration of its RNA-centric mode of action by identifying putative protein partners. For a high-throughput assessment of the PROCA11 interactome, we performed a CRISPR-assisted detection of RNA-protein interactions (CARPID) assay (Yi *et al*, 2020). The CARPID technique leverages the RNA-binding capabilities of BASU-dCasRX to biotinylate proteins located in close proximity to the lncRNA. First, two sets of gRNAs were designed following the protocol of Yi *et al*, considering predicted RNA structures and choosing the longest last exon shared by all three variants as a target sequence. An initial experiment was conducted in hormone-depleted medium, when PROCA11 is physiologically highly expressed in cells (NE-1m). LNCaP-BASU-dCasRX cells were transfected with gRNA-expressing plasmids 24 hours prior to CARPID assay (n=2 replicates for each gRNA set). As a control, we used cells transfected with non-targeting gRNA plasmid (n=6 replicates) (Figure 2A). LC/MS analysis of purified proteins led to identification of 3220 proteins, in total. Following the filtering for unique peptides, fold-change enrichment above 1.5 and p value below 0.1, we isolated 77 protein candidates (Figure 2B).



**Figure 2. Interactome analysis of CARPID candidates revealed different functional clusters.** (A) Schematic representation of CARPID assay and gRNA design on PROCA11; (B) Volcano plot of selected candidates (red) with fold-change above 1.5 ( $p$ -value  $< 0.1$ ); (C) STRING analysis showing protein candidates coimmunoprecipitated with known interactants, thickness of lines indicates the confidence of known interactions.

The refined list of proteins was input into Search Tool for Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk *et al*, 2023) to see if there were established

interactions among the candidate proteins. This could provide clues about PROCA11 association with protein complexes, thus guiding our selection process for single candidate validation. Hence, STRING revealed the possible protein-protein interactions among the CARPID-labelled proteins, and these are distributed into five major functional groups: proliferation, vesicle trafficking, mitochondrial metabolism, RNA processing, and chromatin modifications (Figure 2C). This unveiled PROCA11 association with multiple subunits of the same complex, such as SAGA (USP22, TADA1), spliceosomal (RBM3, GEMIN2, CIRBP), or the ubiquitin ligase (CAND1, CUL5) complexes.

Interestingly, within the group of proteins involved in proliferation, CDKN1B is noteworthy for its key role in halting the G1/S transition of cell cycle by inhibiting the cyclin E/CDK2 and cyclin D/CDK4 complexes. Given that the phenotype we observed with PROCA11 is affecting cell proliferation, CDKN1B stand as a promising candidate for validation. Additionally, STRN and GNAI1 from the same group are of interest, since their association is important for the activation of PI3K-Akt pathway (Lu *et al*, 2004).

Interestingly, there was enrichment of subunits of oxidative phosphorylation (OXPHOS) complexes within the mitochondrial cluster. This included subunits from complex I (NDUFB4, NDUFA12), complex III (UQCRC1), and complex IV (COX4I1). While complexes I, III, and IV are structurally and functionally independent, they make part of a higher structure, supercomplex, known as *respirasome*, whose main function is to create a proton gradient across the inner membrane of mitochondria (Wu *et al*, 2020). Given the intricate connection between OXPHOS activity and cell proliferation, these proteins stand out as prime candidates for experimental confirmation of their association with PROCA11 and how this interaction could proliferation of cells we observed.

Albeit with appropriate caution, several proteins from this initial experiment showed promise as potential PROCA11 interactants, yet they warrant further validation via alternative techniques. First, CARPID should be repeated in cells growing in the presence of the androgen, as this is the condition where we observe the phenotype of PROCA11. Finally, adopting a high throughput complementary approach such as RNA antisense purification coupled with mass spectrometry (RAP-MS), will permit a robust comparison between the two experimental setups and will allow to optimize the selection process for the single candidate validation through subsequent RNA-immunoprecipitation experiments.

### 1.1.3. Discussion

In the vast landscape of cellular biology, lncRNAs have increasingly been recognized as significant modulators of cell homeostasis. Their ability to fine-tune cellular processes has a marked influence on numerous cellular pathways, potentially impacting disease progression, including in cancer. Within this context, my current study has explored the role of a novel lncRNA PROCA11 in the progression and adaptation mechanisms of PCa cells, specifically in response to androgen deprivation. We characterized three different isoforms of this lncRNA (V1, V2, V3) with distinct subcellular distribution, suggesting possible different functions within the cells.

Central to our findings is the observation that PROCA11 overexpression promotes proliferation of LNCaP cells, while its depletion results in an opposite effect. These findings lay the foundation for exploring the mechanistic aspects behind this phenotype.

Approaching the peptide-coding side, the initial CPAT analysis dismissed the likelihood of the PROCA11 variants having a coding potential. Yet, subsequent experiments, particularly the sucrose gradient fractionation, brought to light the potential of the V1 variant to undergo translation. Such result indicates that PROCA11 might play roles beyond the conventionally RNA-centric functions of lncRNAs. However, additional experiments are required to validate these findings and proceed with the functional characterization of the putative peptide.

While probing the potential peptide-coding capacity of PROCA11, we concurrently pursued the exploration of its RNA-centric mode of action by identifying putative protein partners. CARPID assay has facilitated the high-throughput detection of PROCA11 interactome. The interaction with CDKN1B, pivotal in cell cycle regulation, offers a plausible link between PROCA11 and cell proliferation modulation. Furthermore, the association with STRN and GNAI1, which are crucial for PI3K-Akt pathway activation, hints at a more extensive network of signal transduction pathways wherein PROCA11 might play a role. Finally, the presence of OXPHOS complex subunits among the potential interactants of PROCA11 suggests a deeper link between energy metabolism and cellular response to androgen deprivation. The association with components of the respirasome adds an additional layer of complexity, indicating that PROCA11 might modulate mitochondrial activity, thereby influencing cellular energy dynamics and, potentially,

proliferation. Validating the identified protein interactions using complementary techniques will shed light to the undisclosed facets of its functionality.



## 2. The impact of androgen deprivation on AR activity and cellular identity in prostate cancer cells.

### Introduction

AR makes part of the large family of steroid hormone receptors encoded by eight exons on the X chromosome at the locus Xq11-12. Extensive literature describes the AR activity in various cell types and tissues in normal and pathological states (Verhoeven & Swinnen, 1999); (Rana *et al*, 2014), often underlying gender biases (Caggiano *et al*, 2022); (Shukla *et al*, 2016); (Liu *et al*, 2021); (Yoshioka *et al*, 2007); (Lucas-Herald & Touyz, 2022); (Juntti *et al*, 2010); (Schmidt *et al*, 2016b). However, its pivotal function is ultimately linked to normal prostate development and to prostate carcinogenesis (Wu *et al*, 2007). In prostate cancer, AR governs transcription programs to modulate cell signaling, proliferation, apoptosis, adhesion and metabolism, hence, contributing to cell plasticity in epithelial-mesenchymal transition (EMT) (Zhu & Kyprianou, 2010), stemness (Huang *et al*, 2014), neuroendocrine transdifferentiation (NED) (Wright *et al*, 2003a); (Terry *et al*, 2013b) and senescence (Mirochnik *et al*, 2012). In tumor cells the AR gene is often amplified, mutated or producing multiple splice variants AR-Vs, all functional in acquisition of cancer hallmark features (reviewed in Chan *et al*, 2012); (Kwan & Wyatt, 2022); (Jernberg *et al*, 2017). The longest AR variant encoding the 920 amino acids protein of 110 kDa contains four domains: the N-terminal (NTD), the DNA-binding domain (DBD), the hinge region harboring the nuclear localization signal and the ligand binding domain (LBD) (reviewed in Dehm & Tindall, 2007). The LBD and DBD are highly structured whereas the NTD is intrinsically disordered, adopting transient folding to enable recruitment of pioneering factors and co-factors of AR (Yu *et al*, 2020); (Tan *et al*, 2015a). In the canonical ligand-dependent pathway, the inactive AR is retained in the cytoplasm by chaperones, co-chaperones and cytoskeletal proteins (Smith & Toft, 2008). Once bound to a ligand, testosterone or dihydrotestosterone (DHT), via its LBD, AR undergoes conformational change, dimerization, dissociation from the chaperone and translocation into the nucleus. In the nucleus, its DBD binds the androgen-responsive element (ARE) while the transiently folded NTD bridges AR and its partners to the basal transcriptional machinery to activate transcription (Shaffer *et al*, 2004); (reviewed in Chan & Dehm, 2014). Truncated AR-Vs, lacking the LBD and staying constitutively active, maintain their nuclear

residence and transactivation activity sharing most of the canonical target genes with the full-length AR even in the absence of androgen (Chan *et al*, 2012). In addition to transcription activation, AR has been reported to play a role in transcription repression (reviewed in Gritsina *et al*, 2019); (Erdmann *et al*, 2022); (Svensson *et al*, 2014); (Han *et al*, 2019), often achieved by chromatin reorganization at regulatory regions (Zhao *et al*, 2012b); (Cai *et al*, 2011); (Rouleau *et al*, 2002). Remarkably, the repressive function of the AR-V7 variant devoid of the LBD is dramatically disturbed (Erdmann *et al*, 2022). Very recently, nuclear AR has also been reported to regulate co-transcriptionally the alternative cleavage and polyadenylation (Caggiano *et al*, 2022) and splicing of nascent transcripts (Shah *et al*, 2020). Finally, preceding the AR translocation into the nucleus and its transactivation activity, AR can rapidly initiate signal transduction cascades by direct binding to the non-receptor tyrosine kinase SRC1 or the PIK3R1/p85 $\alpha$  subunit of PI3K activating the downstream MAPK/ERK and AKT cascades, respectively (Leung & Sadar, 2017) (Migliaccio, 2000); (Zarif *et al*, 2015); (Baron *et al*, 2004). In turn, MAPK/ERK and PI3K/AKT transactivate AR and induce its translocation to the nucleus, hence, enhancing the ligand-independent nuclear AR signaling pathway (Ueda *et al*, 2002); (Guo *et al*, 2006).

To accomplish all these regulatory functions AR interacts with numerous proteins, including transcription factors, chromatin modifiers, splice factors and others allowing the fine-tune regulation of gene expression in more context-dependent manner (Vélot *et al*, 2021); (Paltoglou *et al*, 2017); (reviewed in Chan *et al*, 2012); (Dahiya & Heemers, 2022). In 1999, for the first time, a steroid receptor RNA coactivator, the SRA1 lncRNA has been reported to interact with AR and stimulate the AR activity. AR is missing a canonical RNA-binding domain, but binds SRA1 in its disordered NTD allowing optimal transactivation of the *TMPRSS2* gene in prostate cancer cells (Lanz *et al*, 1999); (Agoulnik & Weigel, 2009). Very recently, AR's interaction with RNAs has been further explored and extended to other examples (Schmidt *et al*, 2016b); (Schmidt *et al*, 2019b); (Schmidt *et al*, 2020); (daSilva *et al*, 2018a); (Lv *et al*, 2021); (Hsieh *et al*, 2014) revealing a novel non-canonical mechanism modulating the AR activity for the regulation of transcription of specific genes. Notably, Schmidt *et al*. identified a specific pyrimidine-rich motif within SRA1 and another SRA-like long noncoding RNA, named SLNCR, both binding directly the AR NTD in melanoma cells (Schmidt *et al*, 2016b); (Schmidt *et al*, 2020). In the non-invasive melanoma context, SLNCR recruits AR to the EGR1-bound *CDKN1A/p21* promoter

enabling on-to-off switch of transcription. Here, the lncRNA-tethered AR exhibits a negative regulatory function licensing the EGR1 activity (Schmidt *et al*, 2019b). In the invasive melanoma context, SLNCR recruits AR together with the BRN3A transcription factor to the *MMP9* promoter, this time, activating transcription (Schmidt *et al*, 2016b). These two examples are particularly interesting since they illustrate a duality of the AR signaling pathway corroborating with the same RNA but distinct protein partners in both cases contributing to melanoma cancer progression. Remarkably, oligonucleotides blocking the site of interaction either within the RNA molecule or within the AR protein abolish the oncogenic function of SLNCR (Schmidt *et al*, 2020). In addition to SLNCR, other RNAs, belonging to the lncRNA family, contain the aforementioned AR-interacting consensus motif, including HOTAIR that protects AR from proteolysis in the context of castration-resistant prostate cancer (Zhang *et al*, 2015b) but also tethers AR to the *GLI2* promoter to activate its transcription in renal cell carcinoma (Bai *et al*, 2021). The AR NTD is not the only domain reported to bind RNA. The growth arrest specific-5 gene GAS5, another lncRNA with well-known tumor suppressor functions, interacts with the LBD of AR inhibiting its transactivation activity and proliferation of PCa cells (Lv *et al*, 2021). Finally, interaction between the AR protein and RNAs has been addressed transcriptome-wide by RNA-immunoprecipitation of AR following by RNA-sequencing (RIP-seq) in prostate cancer cells in the presence of an androgen analog R1881 (daSilva *et al*, 2018a). This study allowed identification of 619 lncRNAs, named ARA-lincRNAs, which have been proposed to stimulate the transactivation activity of AR *in cis* at AR-regulated neighboring genes by maintaining topologically active domains (TADs) in a close environment. Notably, one such example is an enhancer lncRNA - KLK3e originated from the androgen-responsive enhancer of the *KLK3* gene and facilitating spatial reorganization of the Kallikrein locus to selectively enhance the transcription of the downstream AR-targeted genes *KLK2* and *KLK3* (Hsieh *et al*, 2014), the latter encoding the prostate specific antigen PSA widely used in prostate cancer diagnostics (Cicek *et al*, 2005). In our pursuit to understand the intricate mechanisms driving the evolution of PCa under the conditions of androgen deprivation, we addressed a broader question of cellular plasticity, specifically dissecting AR regulatory circuits and a possible role of RNA in regulation of the AR activity.

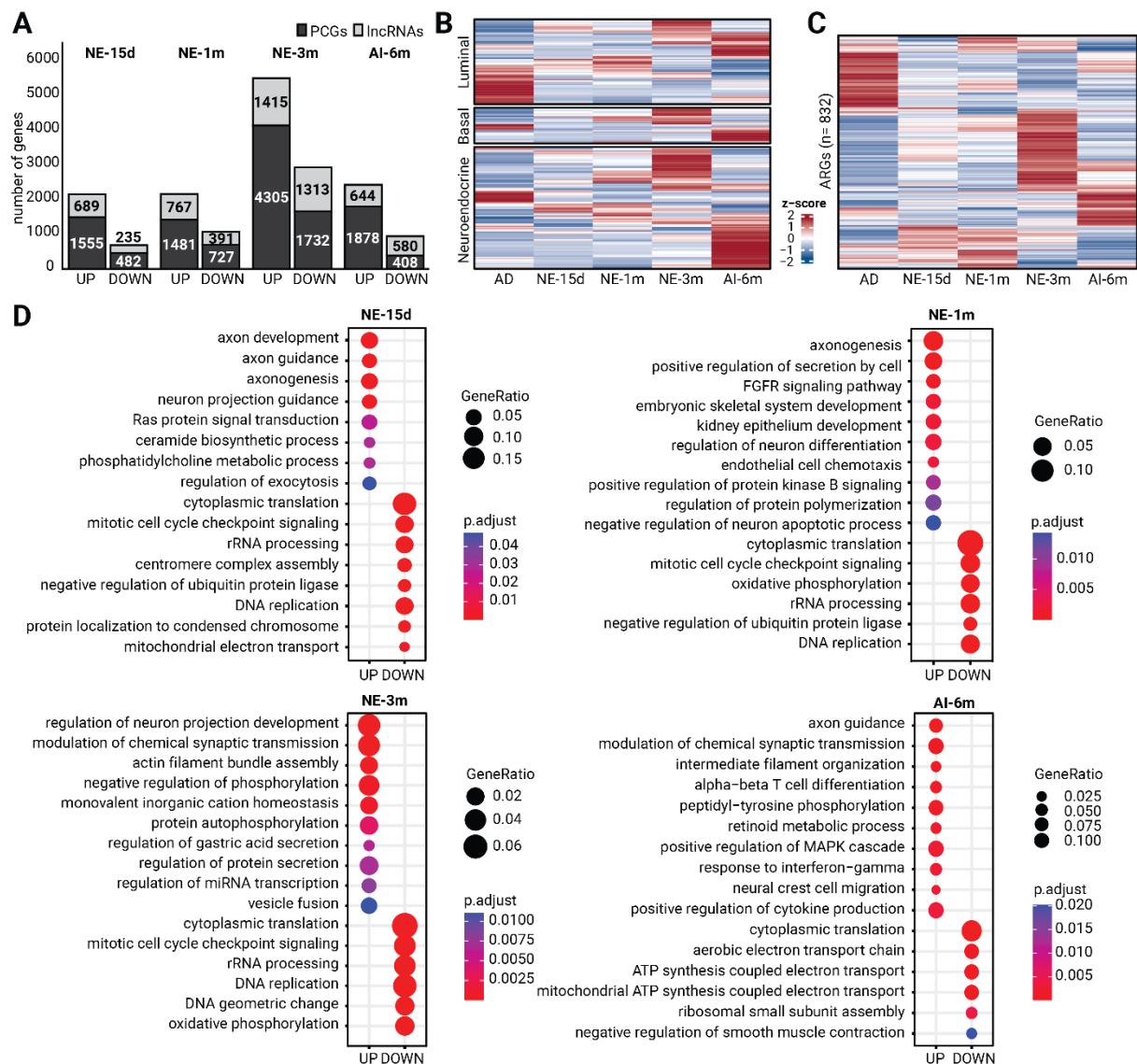
## Results

### **Androgen deprivation induce a loss of luminal gene expression and a gain of basal and neuroendocrine patterns.**

LNCaP cells, which are hormone sensitive, exhibit an epithelial-like morphology while growing in DHT-supplemented medium (AD-cells). With androgen deprivation, these cells experience growth arrest accompanied by an increase in neuroendocrine markers and a transition in morphology toward a neuron-like state, characterized by the emergence of neurites (NE-like). This transition can last up to 6 months, after which the cells revert back to their original epithelial-like morphology and develop androgen-independence (AI), gaining the ability to divide even in the absence of DHT (Terry *et al*, 2013b). Concomitant activation of expression of neuroendocrine markers reported in these cells in response to androgen deprivation suggested an onset of neuroendocrine transdifferentiation (NED) process prior to acquisition of hormone resistance (Terry *et al*, 2013b). To associate these temporal phenotypic alterations with gene expression patterns, we conducted an extensive RNA-sequencing and differential expression analysis of protein-coding genes, comparing the DHT condition to various time points of androgen deprivation. Our analysis revealed that the transcriptome underwent a significant transformation, with an overall activating effect initiating in the early stages of androgen deprivation and persisting until the cells entered the AI stage at 6 months (Figure 1A). We subsequently compared the expression of signatures defining the three primary epithelial cell types in a normal adult prostate (basal, luminal, and neuroendocrine) across AD, NE-like, and AI cells (Henry *et al*, 2018). Androgen deprivation caused a gradual decline in luminal markers and an increase in the expression of basal and neuroendocrine markers (Figure 1B). Late-stage NE-like cells and AI-cells presented a highly mixed pattern, possibly due to the high heterogeneity and plasticity of cells often observed in prostate cancer patients in response to ADT (Chakraborty *et al*, 2023). Functional analysis based on gene ontology indicated an overall decrease in translation-related pathways, while pathways involved in axonogenesis, synapse assembly, and cell growth regulation displayed an enrichment (Figure 1D). It is notable that the AI stage, unlike the intermediate stages, demonstrated an increase in pathways associated with interferon-gamma signaling response, a pathway typically inactive in LNCaP cells (Dunn *et al*, 2005) (Figure 1D). As the main goal of androgen deprivation is to inhibit AR signaling, we decided to track the expression of known androgen-responsive genes (ARGs). Our

analysis confirmed a global release of AR transcriptional activity across all time points, characterized by total reactivation of repressed genes and the silencing of activated genes at NE-3m. Interestingly, some ARGs returned to their original (AD-cells) expression levels after 6 months when cells achieved androgen independence (Figure 1C).

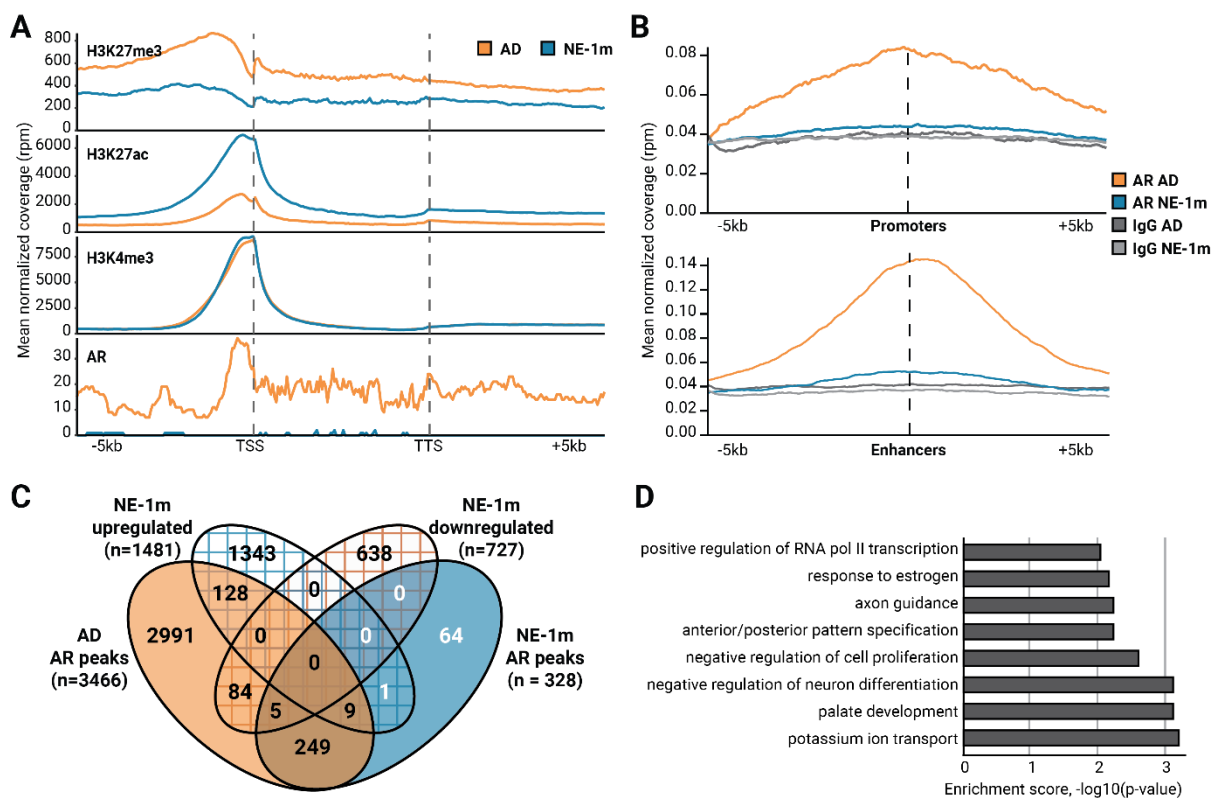
In conclusion, the deactivation of the AR program in LNCaP cells following androgen deprivation is paired with the activation of neurogenesis-related pathways and a remodeling of gene signatures that redefine the cellular identity.



**Figure 1. Transcriptomic changes during androgen deprivation point to a change of cellular identity.** (A) Number of differentially expressed genes (DEGs) at 4 different time points of hormone deprivation (15 days, 1 month, 3 months, 6 months); (B) Heatmap showing unsupervised clustering and gene expression patterns of basal, luminal and NE signatures from (Henry *et al*, 2018); (C) Heatmap showing expression of androgen responsive genes retrieved from (Zhang *et al*, 2018); (D) gene ontology of differentially expressed genes at each time point.

## Androgen deprivation restricts AR recruitment to chromatin.

To elucidate the early changes prompted by androgen deprivation, our study focused on two distinct conditions: LNCaP cells proliferating in the presence of androgen (AD) and NE-like cells cultured for one month in hormone-depleted media (NE-1m). We set out to understand alterations in AR activity caused by androgen deprivation, so we assessed AR recruitment to gene promoters and enhancers by using CUT&RUN. In parallel, histone H3 modifications (H3K27ac, H3K4me3, and H3K27me3) were profiled, enabling us to identify active and repressed regulatory elements. This analysis revealed an overall AR loss from the chromatin alongside a marked decline in repressive marks (H3K27me3), while modifications typical of active chromatin (H3K27ac) increased (Figure 2A). Accordingly, we observed a complete loss of AR at the promoters and enhancers identified by LILY pipeline in the two conditions (Figure 2B).



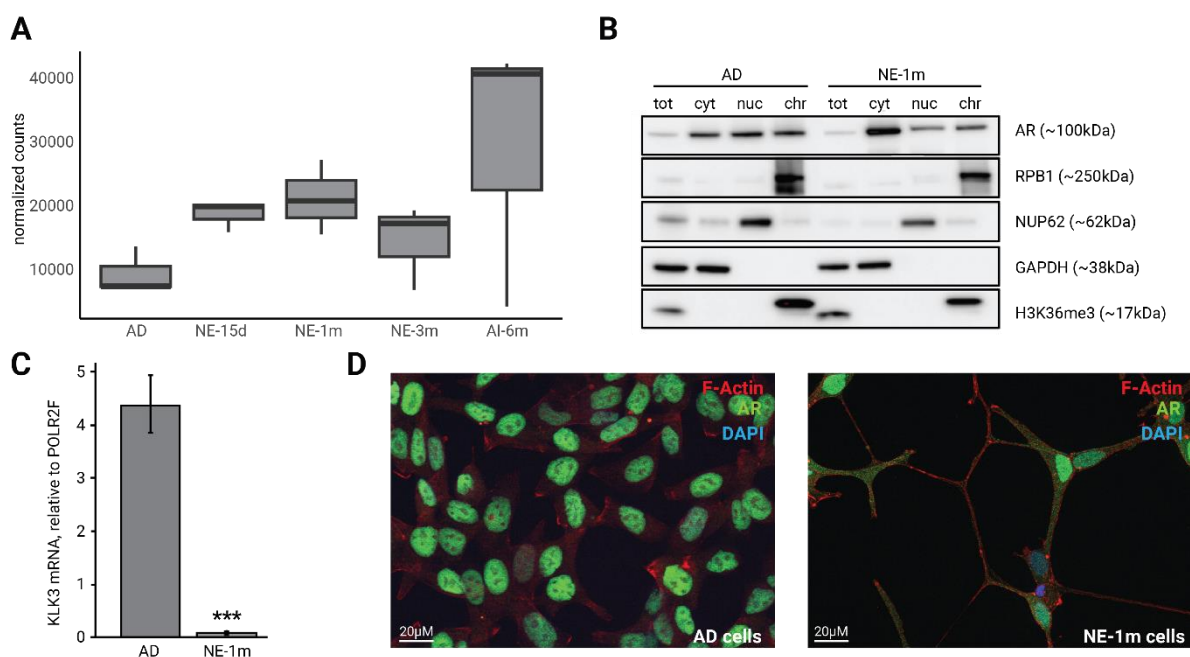
**Figure 2. AR loss from the chromatin is concomitant with chromatin marks of active transcription.** (A) Metagene profiling of H3K27me3, H3K27ac, H3K4me3, and AR of genes that have AR peaks at their promoter; (B) Metagene profiling of AR at enhancers (n=1683) and promoters (n=1476) identified by LILY pipeline in AD and NE-1m conditions; (C) Venn diagrams showing the intersection of AR peaks and differentially expressed protein coding genes in NE-1m; (D) Gene ontology analysis of genes showing increased expression and

These alterations were in agreement with the global derepression of genes upon androgen deprivation observed by RNA-seq (Figure 1A).

We assumed that genes exhibiting AR peaks at their promoters in AD-cells that are upregulated in NE-1m are being repressed by AR (Figure 2C). Intriguingly, gene ontology analysis of these genes revealed an overrepresentation of genes implicated in axon guidance and neuron differentiation (Figure 2D). This supported previous publications reporting the AR repressive activity on the neuroendocrine program (Wright *et al*, 2003b). Notably, PROCA11, the lncRNA identified in my primary project follows the trend of these genes, highlighting its potential contribution to this process.

### AR persists in the nucleus upon hormone depletion of prostate adenocarcinoma cells.

Despite the observed deregulation of ARGs and the loss of AR from chromatin, an upregulation of AR mRNA levels during the NE and AI stages was detected by RNA-seq



**Figure 3. AR persists in the nucleus upon androgen deprivation.** (A) Boxplot showing expression levels of AR mRNA identified by RNA-seq; (B) Western blot visualization in cytoplasm, nucleoplasm and chromatin of AR, RPB1 and H3K36me3 (controls of the nuclear fraction), NUP62 (control of the nucleoplasm fraction), and GAPDH (control of the cytoplasm fraction); (C) RTqPCR quantification of KLK3 mRNA in AD and NE-1m cells; (D) Immunofluorescence imaging of AR (green), F-actin (red, Phalloidin), and DNA (blue, DAPI) in AD- and NE-1m cells.

data (Figure 3A). This unexpected observation prompted a further investigation of the AR protein levels and its subcellular localization. Performing subcellular fractionation and western blot analysis in LNCaP cells under DHT or NE-1m conditions, we discovered an overall decrease in AR levels (two-fold in total protein extracts) and an increase of AR levels in the cytoplasm in NE-1m cells, in contrast to AD-cells. However, we surprisingly noted a persistent presence, at lower levels, of AR in the nucleus and in the chromatin fraction even in the absence of its ligand (Figure 3B). This observation contrasts with the overall loss of AR binding from chromatin and the deregulation of ARGs observed by our CUT&RUN and RNA-seq experiments, respectively, with the typical example of the PSA encoding *KLK3* mRNA (Figure 3C). To validate these findings, we next performed immunofluorescence imaging of AR and confirmed its presence in the nucleus in the absence of androgen (Figure 3D).

In summary, while AR dissociates from chromatin and its transcriptional activity is halted in the absence androgen, AR continues to be expressed and resides within the nucleus. Different ligands and co-factors may contribute to AR translocation and persistence in the nucleus in the absence of androgen, as already suggested (Dagar *et al*, 2019). We aimed to investigate whether and how RNAs contribute to AR activity upon hormone withdrawal.

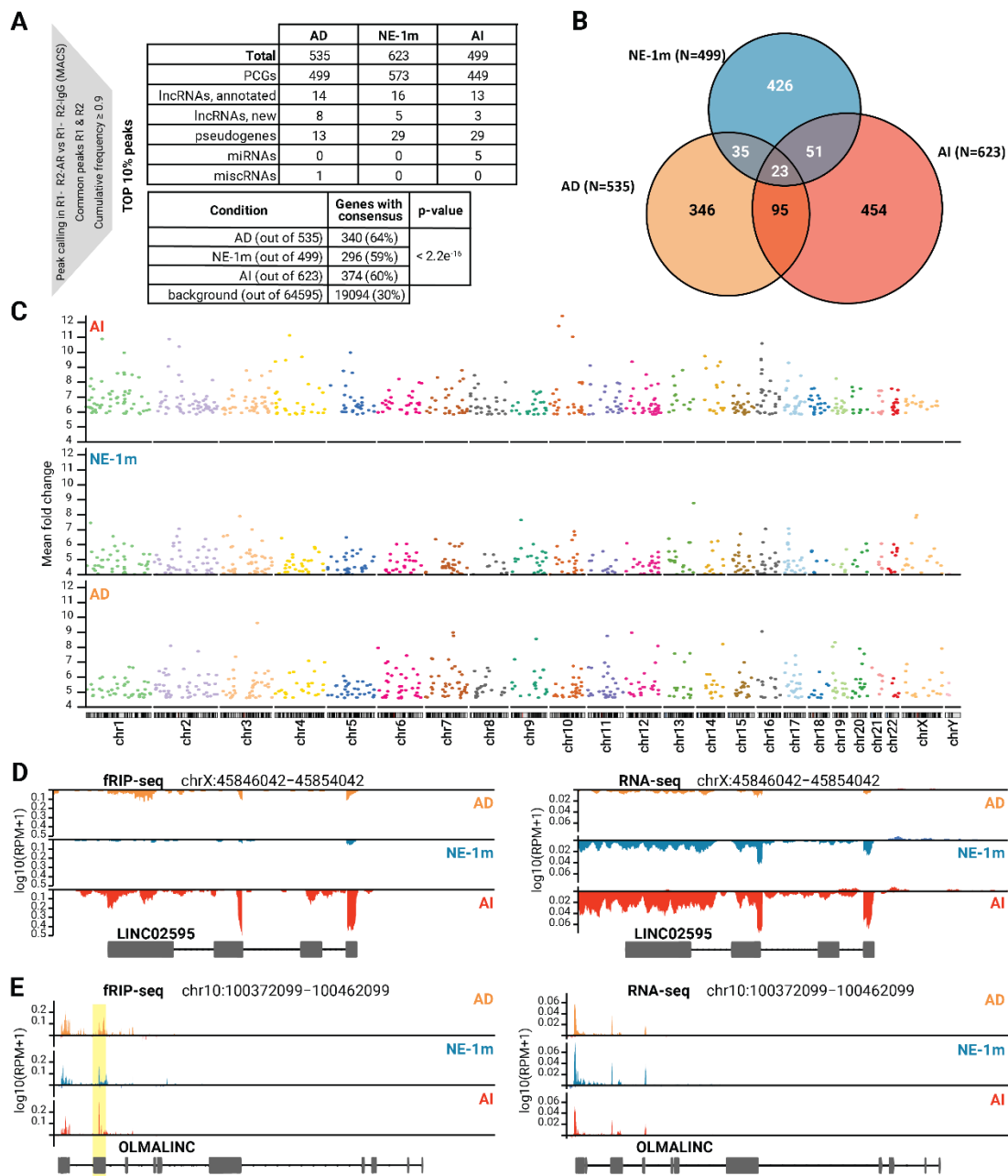
### **AR interaction with RNA is remodeled in response to androgen deprivation.**

To assess AR association with RNAs, we performed crosslinking-mediated RNA immunoprecipitation of AR followed by RNA sequencing (fRIP-seq) at three critical stages of the system: AD, NE-1m, and AI. Immunoprecipitation with IgG was used as control. Data was analyzed using MACS for each strand independently to distinguish the directionality of each peak/transcript. Peaks within the same gene annotation were merged and assigned to the corresponding gene. To increase the stringency, only peaks with averaged fold change above the cumulative frequency of 0.9 were considered for further analysis. This approach identified a total of 1468 RNAs differentially bound to AR across the three stages, with the majority being mRNAs (Figure 4A). More than 59% of these RNAs contained an AR binding consensus sequence (CYUYUCCWS) ( $p\text{-value} < 2.2 \times 10^{-16}$ ) (Figure 4A), as previously identified (Schmidt *et al*, 2020). Comparison of three stages revealed slightly lower number of RNAs bound to AR in NE cells, but remarkably more pronounced AR-RNA associations (more RNAs and higher intensity of peaks) in AI cells



(Figure 4B-C). One lncRNA that we identified to bind AR in AI-cells is LINC02595 (Figure 4D). Previous studies have reported its role in promoting tumor growth in colorectal cancer (Yang *et al*, 2020). Additionally, LINC02595 was identified as functional in a knockdown screening targeting stem cells features within human induced pluripotent stem cells (iPSCs) (Yip *et al*, 2022). In NE cells, the OLMALINC lncRNA gave an intriguing association with AR for one particular exon (Figure 4E). This lncRNA is highly expressed in the frontal cortex white matter and proposed to play a role in oligodendrocytes maturation (Mills *et al*, 2015). It has also been shown to enhance osteogenic-like differentiation in human renal fibroblasts through the interaction and stabilization of the reprogramming factor OCT4 (Zhu *et al*, 2022). To support our findings, we performed the same analysis of the publicly available RIP-seq experiment performed without cross-linking in LNCaP cells treated with androgen analog R1881 (daSilva *et al*, 2018b). This condition resembles our LNCaP cells growing in the presence of DHT, R1881 possessing more affinity to the protein and hence more potent in activation of AR signaling (Chen *et al*, 2006). In total, 957 RNAs were found associated with AR, and 59% containing the AR binding consensus sequence. However, the intersection of two experiments gave rather low overlap of only 30 RNAs in common, 20 containing the consensus motif.

The observed differences might be attributed to the distinct methodologies used, particularly the use of crosslinked versus native conditions. Additionally, there were differences in hormone exposure for LNCaP cells between the studies. In our work, prior to androgen deprivation, we continuously maintained cell culture in 2nM DHT. In contrast, their study exposed cells to 0.1nM R1881 for only 6 hours. Thus, it must be taken into consideration that our cells have adapted to an androgen-supplemented environment. Nonetheless, a more detailed analysis of the transcriptome patterns under both conditions would be required to fully comprehend the differences arising from the two approaches.



**Figure 4. AR interaction with RNA is remodeled in response to androgen deprivation.** (A) Venn diagrams of AR-bound RNAs in AD, NE-1m and AI cells; (B) Table with numbers and percentage of RNA with AR binding motif; (C) Manhattan plot showing the enrichment of AR-RNA association in AD, NE-1m, and AI cells; (D) Snapshots of the reads profiling across the LINC02595 gene obtained by fRIP-seq (left) and RNA-seq (right); (E) Snapshots of the reads profiling across the OLMALINC gene obtained by fRIP-seq (left) and RNA-seq (right). The yellow region highlights the exon enriched in AR-IP.

While these findings are preliminary, they warrant further investigation to understand if RNA binding to AR is important for AR activity. Several questions will be addressed in the continuity of this work. First, we should refine our findings with a similar approach, but

looking at the direct binding of AR to these RNAs. For instance, UV-based crosslinking and immunoprecipitation assay (CLIP) will give us this information and, also, we will be able to see if AR binding is limited to the consensus sequence and if it is enriched in specific regions of the RNA (5' UTR, CDS, 3' UTR). Additional computational analysis will be carried by intersecting these results with CUT&RUN to interrogate whether the interaction takes place at the same locus where AR is recruited or elsewhere within the chromatin environment. We will then focus on few RNA candidates of interest to explore the functional role of this interaction. Thus, single candidate validation by orthogonal techniques, such as RNA pull-down, or imaging (co-IF/FISH or RNA-protein PLA) will help us select potential candidates for further studies. Finally, we will perform gain- and loss-of-function experiments to test whether the interaction has an effect in cells.

## **Discussion**

The AR signaling pathway plays a vital role in healthy prostate development, but when its regulation is disrupted, it can also contribute to the growth and progression of PCa. The common treatment for patients with advanced or metastatic PCa aims to inhibit the AR signaling pathway and androgen production. However, while initially effective, resistance to AR-targeted therapies can emerge, leading to disease progression to CRPC.

This is partly due to the high heterogeneity that PCa tumors display at different levels, as patients may exhibit multiple tumor foci, each at different evolutionary stages, and with specific sensitivity to treatment (Wilkinson *et al*, 2021; Boutros *et al*, 2015; Espiritu *et al*, 2018). One factor that contributes to this heterogeneity is the plasticity of PCa cells, which confers cells high adaptability to treatment and enables them to either dedifferentiate in less “mature” PCa cells, to regenerate the proliferative pool, or to transdifferentiate (e.g. NED)(Qin *et al*, 2012; Wright *et al*, 2003b).

AR plays a pivotal role at governing plasticity of PCa cells thanks to its ability to recruit chromatin modifiers (Heemers & Tindall, 2007; Erdmann *et al*, 2022). Although it is widely recognized for its role as a transcriptional activator, it can also act as a transcriptional repressor through the association with repressive cofactors and complexes such as PRC2 and REST/coREST/LSD1 (Zhao *et al*, 2012a; Cai *et al*, 2014). This repressive activity can be released by the action of AR mutated variants, and it has been

reported to affect genes linked to epithelial-to-mesenchymal transition (EMT) and NED, both determinant to the progression of PCa (Erdmann *et al*, 2022).

In our system we show the transition from hormone-sensitive to NE-like and androgen-independent stage of PCa cells is accompanied with substantial changes to transcriptome with an overall activating effect. Although we detected an increase of AR mRNA levels and persistent nuclear localization, the expression of ARGs changed drastically, showing a global release of AR transcriptional activity, both repressive and activating.

This is also associated with a decline of luminal signature and an increase of basal and neuroendocrine markers, suggesting a change of cellular identity, and possibly reflecting the high heterogeneity and plasticity often observed in patients. At the chromatin level, a global loss of AR was observed along with a decrease of repressive marks (H3K27me3) and increase of active chromatin marks (H3K27ac).

RNA association with transcription factors contribute to their function and is a conserved feature important for vertebrate development and disease (Oksuz *et al*, 2023).

Many studies have shown that this interaction facilitates the fine tuning of gene expression. For example, the lncRNA GAS5 has been shown to act as a “riborepressor” of the Glucocorticoid Receptor (GR) by competing with glucocorticoid responsive elements on the DNA for its binding (Kino *et al*, 2010). This association can also extend the regulation of gene expression beyond transcription. For instance, p53 can negatively regulate its own repressor, MDMX, by suppressing its translation through binding to the 5'UTR of *mdmx* mRNA. Interestingly, this association occurs both in the nucleus and in the cytoplasm (Tournillon *et al*, 2017). While AR lacks an RNA-binding domain, studies have demonstrated that its function can be influenced through interactions with lncRNAs, like SLNCR in melanoma cells (Schmidt *et al*, 2016a, 2019b).

The unexpected finding of AR nuclear persistence in the absence of androgens in our model raised further questions regarding the functionality of its transcription regulatory role during these stages. Thus, we investigated its association with RNAs and if this association could be functional during the transition to the AI stage of PCa cells.

With fRIP-seq, we identified a total of 1468 RNAs associated with AR, some specific to one or shared between the three key stages of the transition. Although it requires further

investigations, we showed that this association with RNA is enhanced at the stage where PCa cells become androgen independent.

Exploring these interactions with additional experiments will allow us to define a link between AR, its transcriptional activity and interaction with RNA in the onset of the androgen independent stage.

## **Material and Methods**

### **Cell culture**

The LNCaP cell line was purchased from ATCC (CRL-1740), cultured at 37°C in a humid 5% CO<sub>2</sub> atmosphere in either the RPMI 1640+GlutaMAX medium (Life Technologies) supplemented with 10% fetal bovine serum and 2 nM DHT (DHT-LNCaP) or the phenol red-free RPMI 1664+GlutaMAX medium supplemented with 10% dextran charcoal-stripped FBS (NE-LNCaP and AI-LNCaP).

### **Subcellular fractionation**

The protocol was adapted from (Henry *et al*, 2018) for 10<sup>7</sup> cells. All steps were performed on ice with ice-cold buffers supplemented with 20 U/μl SUPERase-IN (Thermo Fisher Scientific) for RNA extraction and 0.1 mM AEBSF (SIGMA) for protein extraction. RNA extraction from all fractions was performed using TriZol (Thermo Fisher Scientific) at 65°C for 5 minutes with regular vortexing and then purified with miRNeasy kit (Qiagen) according to the manufacturer's instructions. For protein extractions, the chromatin fraction was sonicated for 8 min (30s-on/30s-off, "high power") on the Bioruptor Plus (Diagenode). Proteins' content was quantified with the Thermo Fisher Scientific™ BCA Protein Assay Kit (Thermo Fisher Scientific).

### **Polysome isolation by sucrose gradient**

The protocol from Baudin-Baillieu A. et al., 2016 was used for 2x10<sup>7</sup> LNCaP cells per condition, in the presence of DHT and hormone depleted for 72 hours. Prior fractionation, cells were treated with 10ug/mL puromycin for 30' at 37°C 5% CO<sub>2</sub>. Following, cycloheximide (100ug/mL) was added to the culturing medium for 5 minutes.

### **Cross-link mediated RNA immunoprecipitation (fRIP)**

The protocol was adapted from (G Hendrickson *et al*, 2016) for  $20^7$  cells per condition and using 6ug of AR (Abcam ab108341) or IgG (Abcam ab37415) antibody per condition. After the reverse-crosslinking step RNA was extracted following the procedure of the miRNeasy kit (Qiagen) including the DNase treatment step.

### **fRIP-seq library preparation and sequencing**

Libraries were prepared by SMARTer Stranded Total RNA-seq Kit-Pico Input Mammalian using 10ng of RNA. Libraries were sequenced on a NovaSeq – S1 device in a pair-end 100 bp read length mode.

### **fRIP-seq analysis**

Unique mapped reads (with a tag NH:i:1) mapped to plus and minus strands were separated using a command "samtools view" of samtools 1.9. Peaks were called for each strand separately using a "callpeak" command of MACS v3.0.0b1 with parameters " --broad --broad-cutoff 0.05", for each IP and its associated IgG. Shared peaks between replicates of a given condition were merged and combined if overlapping the same gene annotation with associated averaged fold change and p-values.

### **Protein extraction and western blot**

Cells at 80% of confluence were lysed in RIPA buffer (Thermo Scientific) supplemented with 0.1 mM AEBSF (SIGMA). Total of 20 µg of the whole protein extract was separated on a NuPAGE Novex 4-12% Bis-Tris Protein Gel, transferred using Thermo Fisher Scientific iBlot Dry Blotting System (Thermo Fisher Scientific) and blotted against GAPDH (Millipore, mab374, 1:5000 dilution), AR (Abcam, ab108341, 1:1000), overnight at 4 °C, then washed and incubated with a corresponding HRP-conjugated secondary antibody (1:5000 dilution) for 1 hour at room temperature and visualized using the SuperSignal West Femto, Dura or Pico Substrate (Thermo Fisher Scientific). Quantifications of protein amounts were performed relative to GAPDH using the ImageJ software.

### **Immunofluorescence**

A total of 20000 cells suspension was seeded on a 4-well microscope slide and let growing for 24 hours. Following PBS wash, cells were fixed using Formaldehyde(3%)-

Sucrose(2%) solution for 15 minutes, permeabilized and blocked with 10% goat serum (Thermo Fisher Scientific). For F-actin staining, cells were incubated with 0.5 µg/ml DMSO solution of phalloidin-tetramethylrhodamine isothiocyanate (TRITC) (Sigma) for 40 minutes at room temperature. For AR, cells were incubated with the primary antibody (1:100 dilution, Abcam, ab108341, LOT:GR3414581-3) for 40 minutes at 37°C, washed in PBS and incubated with Alexa Fluor 594 Goat anti-Rabbit secondary antibody (1:400, Thermo Fisher Scientific, A11037) for 30 minutes at 37°C. Slides were washed and mounted using a ½ dilution of Vectashield Mounting Medium with DAPI (Vector Laboratories).

### **CUT & RUN**

The experiment was performed in two replicates following the protocol from Henikoff lab with modifications described in (Skene *et al*, 2018). Antibodies against H3K27ac (Millipore, MABE647, 1:50), H3K27me3 (Cell signaling, C36B11, 1:50), H3K4me3 (Millipore, 04745, 1:50); AR (Abcam, ab108341, LOT:GR3414581-3), DNA fragments were purified with NEB Monarch PCR & DNA purification kit following the protocol enriching for short DNA fragments and was quantified by Qubit HS DNA kit.

### **CUT & RUN libraries preparation and sequencing**

CUT & RUN libraries were prepared using the TruSeq ChIP-Sample Prep Kit-Set A and PCR Box (Illumina) from 5 ng of DNA with 10 PCR cycles for amplification following the manufacture's procedures. Concentration and quality were assessed by Qubit with dsDNA HS Assay Kit (Thermo Fisher Scientific) and High Sensitivity DNA Analysis Chip Kit (Agilent Technologies). Libraries were sequenced on a NovaSeq – S1 device in a pair-end 100 bp read length mode.

### **CUT & RUN analysis**

Reads were first aligned to human genome version GRCh38 using bowtie (version 2.4.1) (Langmead & Salzberg, 2012), with options -I 10 -X 700 --no-mixed. Reads with MAPQ value  $\geq 30$  were kept for further analysis. Peaks were called relative to IgG control using MACS2 version 2.2.6 (Zhang *et al*, 2008) and options : -f BAMPE -g hs -B --keep-dup all for AR, H3K4me3 and H3K27ac or - MACS2 version 2.2.6 ; options : -f BAMPE -g hs -B --keep-dup all --broad --broad-cutoff 0.05 for H3K27me3, pooling duplicate reads. For further

analysis, common peaks between conditions were also merged to peak regions. The merged peaks were assigned to closest genes and the shortest distance between each peak and the closest transcript start site (TSS) coordinates were calculated using Gencode v32 annotation. Regulatory elements (promoters, enhancers and super-enhancers) were annotated using the LILY pipeline (Boeva *et al*, 2017). Peaks overlap with genomic elements was retrieved from genome mapping information of Gencode transcripts using BEDtools.

### AR binding sites search

In total, 9 different AR binding sites were retrieved from the literature and are listed in the Table:

Description	ID	Motif sequence	Number of sites (%)		Ref
			+DHT	NE-like	
Canonical	<b>ARE 1</b>	AGAACAN(3)TGTTCT	5 (0.2%)	0	(Wang <i>et al</i> , 2007)
Head-to-head_short	<b>ARE 2</b>	AGAACAN(0,2)TGTTCT	3 (0.12%)	1 (0.61%)	(Wang <i>et al</i> , 2007)
Head-to-head_long	<b>ARE 3</b>	AGAACAN(4,8)TGTTCT	1 (0.04%)	0	(Wang <i>et al</i> , 2007)
Tail-to-tail	<b>ARE 4</b>	TGTTCTN(0,8)AGAACA	6 (0.23%)	0	(Wang <i>et al</i> , 2007)
Direct_repeats	<b>ARE 5</b>	AGAACAN(0,8)AGAACA	7 (0.27%)	2 (1.22%)	(Wang <i>et al</i> , 2007)
Halfsite_ARE	<b>ARE6</b>	AGAACA	195 (7.61%)	20 (12.2%)	(Wang <i>et al</i> , 2007)
ARE_HOMER	<b>ARE7</b>	AGAACAGNCTGTTCTT	549 (21.41%)	13 (7.93%)	(Heinz <i>et al</i> , 2010)
halfsite_HOMER	<b>ARE8</b>	CCAGGAACAG	1 797 (70.09%)	128 (78.05%)	(Heinz <i>et al</i> , 2010)



PromoV3	<b>ARE9</b>	GGACAAGGA	1 (0.04%)	0	(Farré <i>et al</i> , 2003) (Messeguer <i>et al</i> , 2002)
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### **RNA extraction and reverse transcription**

Total or AR-immunoprecipitated RNA extraction was performed directly from cell cultures with miRNeasy kit according to the manufacture' instructions including the DNase treatment step (Qiagen). Reverse Transcription (RT) was performed on 500 ng of RNA with random primers and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific).

### **Quantitative PCR**

cDNA was diluted 10-50 times and quantified by real-time PCR with LightCycler® 480 SYBR Green I Master (SIGMA). The *RPL11* gene was used as a reference to quantify a relative abundance of each cDNA. Error bars represent standard deviations in relative quantity of cDNA prepared from at least three independent experiments.

### **Gene features analysis**

Gene ontology (GO) biological process, KEGG pathways and cellular compartment terms enrichment analyses were performed using DAVID (Huang *et al*, 2008); (Huang *et al*, 2009). The gene enrichment score was calculated as  $-\log_{10}(P\text{-value})$ .

### III. Conclusions

The main objective of my thesis research was to uncover the role of lncRNAs in response to androgen deprivation in PCa cells, with a particular emphasis on NED. Utilizing the LNCaP in dynamic vitro system, we could capture, transcriptome-wise, the transitions that lead to NED and androgen-independent growth. To explore the role of lncRNAs involved in NED we focused on the first time points of this transition and could identify 19 candidates, of which 15 are unannotated lncRNAs, named PROCAs.

PROCA11 emerged among the candidates as the most induced lncRNA during NED transition. In-depth characterization of PROCA11 revealed three unique isoforms with diverse subcellular localizations, suggesting different fate and function. Intriguingly, the transcription of PROCA11 is controlled by AR repressive activity, and this restraint is lifted upon hormone depletion, leading to increased expression levels of PROCA11.

In the context of androgen deprivation, the cellular landscape undergoes significant alterations. The absence of a key growth factor culminates in suppressed proliferation and the equilibrium between apoptotic and anti-apoptotic signals tilts toward the programmed cell death. Concurrently, the regulatory scope of AR on the transcriptome diminishes, as we have shown with the extensive deregulation of ARGs in our RNA-seq. The upregulation of PROCA11 in this scenario represents a conundrum. While ectopically introduced PROCA11 enhances cell proliferation, its endogenous expression following the androgen depletion is associated with a state in which cells are quiescent. By contrast, the depletion of the already low basal levels of PROCA11 in cells in the presence of DHT hindered their proliferation. It is conceivable that the diverse molecular landscapes, in which we have the ectopic expression and the endogenous one, provide distinct interactomes of PROCA11 that dictate its divergent impact on cells.

On the other hand, also the investigation of AR activity gave us some insights. RNA sequencing analysis showed a global deregulation of ARGs after hormonal deprivation, confirming the release of AR transcriptional influence. This is accompanied with a loss of luminal gene signature and an upregulation of basal and neuroendocrine markers, underscoring the adaptability and plasticity of these cells. It is worth mentioning that a

similar effect on gene signature also occurs when we deplete PROCA11 in AD cells, suggesting a role of our lncRNA in this process.

In accordance with what we observed at the transcriptomic level, chromatin-based investigations using CUT&RUN revealed a loss of AR occupancy at gene promoters and enhancers in the hormone-deprived state. Intriguingly, gene ontology analysis of a subset of genes, under AR's repressive domain and upregulated in NE-1m, showed an enrichment in pathways like neuron differentiation, axonogenesis, and development. Such an alignment of gene ontologies with those identified in the global transcriptome hints at AR's potential role in holding back cellular plasticity programs, including neuroendocrine differentiation. A similar scenario was reported in a recent study (La Rosa *et al*, 2021), in which the activation of AR augmented the pool of neural progenitor cells by limiting their propensity to differentiate into neurons.

However, an intriguing discrepancy emerges when analyzing AR's subcellular distribution. Immunofluorescence studies distinctly show a remarkable nuclear retention despite the evident transcriptomic inactivity of AR in the post-hormonal deprivation state. This observation, further corroborated by subcellular fractionation, raises pertinent questions about AR's functional role within the nucleus under these conditions.

The pronounced nuclear localization of AR, despite its transcriptomic dormancy, led to a deeper probe into its potential interactions. Utilizing RIP-seq, the associations between AR and various RNA molecules during different stages of prostate cancer progression were mapped. Interestingly, AR showed a preference for mRNAs over the expected lncRNAs. This RNA-binding landscape of AR shifts as cells undergo post-hormonal deprivation, hinting at a dynamic evolution of AR-RNA relationships. As the cells entered the androgen-independent phase, a marked increase in AR's RNA associations was observed. This presents an intricate relationship wherein RNA might either stabilize AR's nuclear localization, or AR may influence post-transcriptional regulation of these associated RNA molecules. This intricate relationship highlights the multifaceted roles of AR beyond traditional gene transcription regulation, potentially unveiling new avenues for therapeutic interventions in prostate cancer progression.

Ultimately, this thesis provides a comprehensive understanding of AR's role alongside lncRNAs during androgen deprivation, serving as a foundation for further inquiries while enriching our molecular perspective on prostate cancer evolution.

## IV. References

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## RÉSUMÉ

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L'axe de signalisation du récepteur des androgènes (RA) est crucial pour le développement du cancer de la prostate, traité principalement par des thérapies de privation d'androgènes (ADT). Cependant, le cancer de la prostate résistant à la castration (CRPC) émerge souvent, présentant des caractéristiques neuroendocrines. Les longs ARN non codants (lncARN) contribuent à la tumorigénèse, y compris pour l'apparition du CRPC. En utilisant un système cellulaire dynamique *in vitro*, ce projet a pour but d'identifier et d'explorer les lncARN dans la différenciation neuroendocrine (NED) lors de l'apparition du CRPC et d'étudier l'activité AR sous ADT. L'étude du transcriptome a permis d'identifier 15 nouveaux lncARNs, dont PROCA11, l'un des plus précoces et le plus régulé en cas de privation d'androgènes. Les altérations de l'expression de PROCA11 affectent la prolifération des cellules. Corroborant ces observations, des essais d'interaction ARN-protéine ont permis d'identifier plusieurs protéines associées à PROCA11 et impliquées dans la régulation de la prolifération et de l'homéostasie cellulaire. L'activation de la transcription de PROCA11 est associée à une dérégulation globale des gènes réagissant aux androgènes, indépendamment de la persistance de RA dans le noyau en cas de privation prolongée d'hormones. Le profilage chromatinien de RA et des modifications d'histones ont confirmé la dissociation de RA de la chromatine et un large remodelage du paysage épigénétique au cours de la NED. L'immunoprécipitation de RA en présence et en absence d'androgène a révélé son association avec des ARN spécifiques, suggérant un rôle possible de ces derniers dans la signalisation de RA dans des contextes dépendants ou pas de l'androgène.

## MOTS CLÉS

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Long ARN non codants, récepteur des androgènes, neuroendocrine, cancer de la prostate

## ABSTRACT

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The Androgen Receptor (AR) signaling axis is crucial for prostate cancer development, treated primarily with androgen deprivation therapies (ADT). However, castration-resistant prostate cancer (CRPC) often emerges, showing neuroendocrine features. Long non-coding (lnc)RNAs contribute to tumorigenesis, including CRPC onset. By using an *in vitro* dynamic cell system, this project aimed to identify and explore lncRNAs in Neuroendocrine Differentiation (NED) in CRPC onset and to investigate the AR activity upon ADT. Transcriptome study identified 15 novel lncRNAs including PROCA11, one of the earliest and most up-regulated gene upon androgen deprivation. Alterations of PROCA11 expression affect proliferation in cells. Supporting the observed cellular functions, RNA-protein interaction assays identified several proteins associated with PROCA11 and involved in regulation of proliferation and cellular homeostasis. Activation of PROCA11 transcription is associated with overall deregulation of androgen-responsive genes regardless AR persistence in the nucleus upon prolonged hormone deprivation. Chromatin profiling of AR and histone modifications confirmed the dissociation of AR from the chromatin and a broad remodeling of the epigenetic landscape during NED. RNA immunoprecipitation of AR in the presence and absence of androgen revealed its association with specific RNAs, suggesting a possible role of RNAs in AR signaling in the androgen-dependent and independent context.

## KEYWORDS

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Long non-coding RNA, Androgen Receptor, neuroendocrine differentiation, prostate cancer, castration-resistant prostate cancer