Universitat Autònoma de Barcelona

Dual inhibition of KIF11 and the Androgen Receptor axis as a novel therapeutic approach for Castration Resistant Prostate Cancer

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ABSTRACT

Prostate cancer (PCa) is the second most common type of cancer and the fifth cause of cancer-related deaths among men, with almost 1.4 million of new cases and more than 375000 deaths worldwide in 2020. Most PCa related deaths occur after androgen-deprivation therapy (ADT) failure, when the tumor evolves to castration resistant prostate cancer (CRPC). Androgen receptor (AR) targeted therapies, such as enzalutamide, have shown to prolong CRPC patient survival, but most tumors become resistant through several mechanisms often related with the AR signaling pathway. Taxanes are a class of antimitotic agents that target microtubules and are the only chemotherapeutic drugs that have demonstrated clinical benefits after the development of AR-targeted therapy resistance. However, their related severe side effects and the development of resistance limit their clinical utility, leaving few therapeutic options to CRPC patients. Here we showed that overexpression of Kinesin Family Member 11 (KIF11), a motor protein essential for bipolar spindle formation and mitosis, is related with PCa progression, poor prognosis, the development of CRPC and hormonal therapy resistance. Inhibition of KIF11 with 4SC-205, a novel oral small-molecule inhibitor, effectively inhibits PCa growth in several preclinical models *in vitro, ex vivo* and *in vivo.* Moreover, based on the results of several gain and loss of function assays, we propose novel mechanistic insights based on a positive feed-back loop between the AR and KIF11 that could be contributing to PCa progression. Also, we demonstrated that the combination of KIF11 and AR inhibition by 4SC-205 and the AR inhibitor enzalutamide respectively, synergistically inhibits CRPC growth both, *in vitro* and *in vivo*. Altogether, our results indicate that KIF11 plays an important role in the development of CRPC and endocrine therapy resistance and we provide a good rationale for the development of a therapeutic strategy based on the combination of 4SC-205 and enzalutamide for the treatment of this lethal disease.

Key words: KIF11, prostate cancer, CRPC, androgen receptor, mitosis, mitosis-targeted therapy

RESUMEN

El cáncer de próstata es la segunda neoplasia más frecuente y la quinta causa de mortalidad por cáncer en hombres. En 2020 se diagnosticaron alrededor de 1.4 millones de nuevos casos y dio lugar a más de 375000 muertes en todo el mundo. La mayoría de estas muertes se producen cuando el tumor desarrolla resistencia a la terapia de deprivación de andrógenos, estadio que se conoce como cáncer de próstata resistente a la castración (CPRC). En este punto, los pacientes son tratados con inhibidores del receptor de andrógenos, como la enzalutamida, que han demostrado aportar beneficios clínicos a este grupo de pacientes. Desafortunadamente, la mayoría desarrollan resistencias tras menos de dos años de tratamiento. Se han descrito múltiples mecanismos implicados en este proceso, muchos de ellos relacionados con alteraciones que afectan al receptor de andrógenos. Los taxanos son agentes quimioterapéuticos que inhiben la mitosis celular interfiriendo con los microtúbulos y son los únicos compuestos que han demostrado aportar un beneficio clínico a los pacientes resistentes a los antagonistas del receptor de andrógenos. A pesar de ello, los graves efectos secundarios asociados a su uso junto con la inevitable aparición de resistencias limitan su utilidad en la práctica clínica. En este trabajo hemos observado que la sobreexpresión de “Kinesin Family Member 11” (KIF11), una proteína mitótica clave durante la formación del huso acromático, está relacionada con la progresión del cáncer de próstata, mal pronóstico y con el desarrollo de CPRC y resistencia a la terapia hormonal. La inhibición de esta proteína con 4SC-205, un nuevo y específico inhibidor de uso oral, mostró una alta eficacia inhibiendo la viabilidad de varios modelos preclínicos de cáncer de próstata, tanto *in vitro, ex vivo* como *in vivo.* Además, a partir de los resultados obtenidos tras una serie de ensayos de pérdida y ganancia de función, proponemos que existe un mecanismo de retroalimentación entre el receptor de andrógenos y KIF11 que podría estar favoreciendo a la progresión del cáncer de próstata. Finalmente, demostramos que la doble inhibición KIF11 y del receptor de andrógenos, usando 4SC-205 y enzalutamida respectivamente, es capaz de inhibir de manera sinérgica el crecimiento tumoral *in vitro* e *in vivo*. Por todo ello, estos resultados indican que KIF11 juega un papel importante en el desarrollo de CPRC y resistencia a terapias hormonales. Además proponemos un mecanismo que daría sentido al desarrollo de una estrategia terapéutica basada en el uso de 4SC-205 y la enzalutamida, del que se podrían beneficiar este grupo de pacientes, hoy en día aun con pocas opciones.

Palabras clave: KIF11, cáncer de próstata, CPRC, receptor de andrógenos, terapia dirigida antimitosis

RESUM

El càncer de pròstata és la segona neoplàsia més freqüent i la cinquena causa de mortalitat per càncer en homes. El 2020 es van diagnosticar al voltant de 1.4 milions de nous casos i va donar lloc a més de 375.000 morts a tot el món. La majoria d'aquestes morts es produeixen quan el tumor desenvolupa resistència a la teràpia de deprivació d'andròg ens, estadi que es coneix com a càncer de pròstata resistent a la castració (CPRC). En aquest punt, els pacients són tractats amb inhibidors del receptor d'andròg ens, com l'enzalutamida, que han demostrat aportar beneficis clínics a aquest grup de pacients. Desafortunadament, la majoria de pacients desenvolupen resistències. S'han descrit diversos mecanismes implicats en aquest procés i en la majoria de casos estan relacionats amb alteracions al receptor d'andròg ens. Els taxans són agents quimioterapèutics que inhibeixen la mitosi cel·lular interferint amb els microtúbuls i són els únics compostos que han demostrat aportar un benefici clínic als pacients resistents als antagonistes del receptor d'andròg ens. Tot i això, els greus efectes secundaris associats al seu ús juntament amb la inevitable aparició de resistències, limiten la seva utilitat a la pràctica clínica. En aquest treball hem observat que la sobreexpressió de “Kinesin Family Member 11” (KIF11), una proteïna mitòtica clau durant la formació del fus acromàtic, està relacionada amb la progressió del càncer de pròstata, mal pronòstic i amb el desenvolupament de CPRC i resistència a la teràpia hormonal. La inhibició d'aquesta proteïna amb 4SC-205, un inhibidor d'ús oral nou i específic, ha mostrat una alta eficàcia inhibint la viabilitat de diversos models preclínics de càncer de pròstata, tant *in vitro*, *ex vivo* com *in vivo*. A més, a partir dels resultats obtinguts després d'una sèrie d'assajos de pèrdua i guany de funció, proposem que hi ha mecanismes de retroalimentació entre el receptor d'andròg ens i KIF11 que podria estar afavorint la progressió del càncer de pròstata. Finalment, demostrem que la combinació de la inhibició de KIF11 i del receptor d'andròg ens, usant 4SC-205 i enzalutamida respectivament, és capaç d'inhibir de manera sinèrgica el creixement tumoral *in vitro* i *in vivo*. Per tot això, aquests resultats indiquen que KIF11 juga un paper important en el desenvolupament de CPRC i resistència a teràpies hormonals. A més, proposem un mecanisme que donaria sentit al desenvolupament d'una estratègia terapèutica basada en l'ús de 4SC-205 i l'enzalutamida, del qual es podrien beneficiar aquest grup de pacients, avui encara amb poques opcions.

Paraules clau: KIF11, càncer de pròstata, CPRC, receptor d'andròg ens, teràpia dirigida antimitosi

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Abbreviations:

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3D → Three-dimensional

A

AD → Androgen dependent

ADT → Androgen depravation therapy

AI → Androgen independent

APC → Antigen-presenting cells

AR → Androgen receptor

ARE → Androgen response elements

ARTA → androgen receptor pathway targeting agents

AR-Vs → AR variants

AS → Active surveillance

ATCC → American Type Culture Collection

AURKs → Aurora kinases

AURKA → Aurora Kinase A

AURKB → Aurora Kinase B

AURKC → Aurora Kinase C

B

BASS → Bipolar assembly

BC → Breast cancer

BCR → Biochemical recurrence

BPH → Benign prostatic hyperplasia

C

CDK → Cyclin-dependent kinases

CENPE → centromere-associated protein E

ChIP → Chromatin immunoprecipitation

CHX → Cycloheximide

CI → Combination indexes

CIN → Chromosome instability

Co-IP → Co-immunoprecipitation

CPC → chromosome passenger complex

CRPC → Castration resistant prostate cancer

CSS → Charcoal stripped serum

D

DBD → DNA binding domain

DDR → DNA damage response

DHT → Dihydrotestosterone

DMSO → Dimethyl sulfoxide

DRE → Digital rectal examination

DST → Dose-limiting toxicity

E

EAU → European Association of Urology

EBRT → External-beam radiation therapy

ER → Estrogen receptor

ETS → E26 transformation-specific

F

FBS → Fetal bovine serum

FFPE → Formalin-fixed paraffin-embedded

G

G-CSF → Granulocyte colony-stimulating factor

GO → Gene ontology

GR → Glucocorticoid receptor

GS → Gleason Score

H

H3 → Histone 3

H/E → Hematoxylin and eosin

HGPIN → High grade prostatic intraepithelial neoplasia

HIF-1α → Hypoxia-inducible factor 1

HRR → Homologous recombination repair

HSP → heat shock proteins

I

IC_{50} → Half-maximal inhibitory concentration

IHC → Immunohistochemistry

IMiDs→ Immunomodulatory agents

IP → Immunoprecipitation

ISUP → International Society of Urological Pathology

K

KIF → Kinesin superfamily

KIF11 → Kinesins family member 11

KSP → Kinesin spindle protein

L

LBD → Ligand-binding domain

LHRH → Luteinizing hormone releasing hormone

LNP → Lipid nanoparticles

LRP → Laparoscopic radical prostatectomy

M

mCRPC → Metastatic castration resistant prostate cancer

mCSPC → metastatic castration sensitive prostate cancer

min → Minutes

MM → Multiple myeloma

MMR → Mismatch repair

mp-MRI →Multiparametric-magnetic resonance imaging

MPS1 → Monopolar spindle protein 1

MSI → Microsatellite instability

MT → Microtubules

MTA → Microtubule targeting agents

MTD → Maximum tolerated dose

N

NEK → NIMA-related kinases

nmCRPC → Non-metastatic castration resistant prostate cancer

O

ORR → Overall response rate

P

PAP → Prostatic acid phosphatase

PARP → Poly(ADP-ribose) polymerase

PBS → Phosphate-buffered saline

PCa → Prostate cancer

PD-1 → Programmed death-1

PDOX → Patient-derived orthotropic xenograft

PDX → Patient-derived xenograft

P-gp → P-glycoprotein

pH3 → Phosphorylated-Histone H3

PI → Propidium iodide

PI → Proteasome inhibitors

PIN → Prostatic intraepithelial neoplasia

PLK1 → Polo-like kinase 1

PR → Partial response

PSA → Prostatic specific antigen

R

RARP → Robotic assisted radical prostatectomy

RRMM → Recurrent/refractory multiple myeloma

RT → Radiotherapy

S

SAC → Spindle assembly checkpoint

SD → Stable disease

sec → Seconds

siRNAs → Small interfering RNAs

T

TBP → TATA-box binding protein

TNM → Clinical Tumour Node Metastasis

TURP → Transurethral resection of the prostate

U

UICC →Union for International Cancer Control

US-guided biopsy → Ultrasound guided biopsy

US-MRI → Ultrasound-magnetic resonance imaging

V

VEGF → Vascular endothelial growth factor

VGPR → Very good partial response

1. Introduction

1.1. The prostate gland

1.1.1. Anatomy and histology of the human prostate

The human prostate is a tubuloalveolar exocrine gland that belongs to the male reproductive system. It is located in the subperitoneal compartment, beneath the bladder and in front of the rectum, surrounding the urethra [1].

The prostate gland is implicated in several functions, being among the most important the secretion of small molecules to the seminal plasma contributing to fertility, providing nutritional support and an alkaline environment to the sperm. Due to its muscular characteristics, it is also important for the control of urine output from the bladder and seminal fluid through the urethra during ejaculation. Moreover, it also participates in the metabolism of testosterone to dihydrotestosterone (DHT), a more potent androgen [2].

Histologically, the prostate has been divided into four different anatomical regions, the peripheral, central, fibromuscular and transitional zone, as proposed by McNeal in the early 1980s [3]. As shown in **Figure 1**, the peripheral zone is placed in the outer portion of the prostate and is the site of origin of around 70% of prostatic neoplasias [4]. The transitional zone is located near the urethra and it is enlarged in the majority of older men giving rise to benign prostatic hyperplasia (BPH), a common benign proliferation of the transitional zone tissue [1,5].

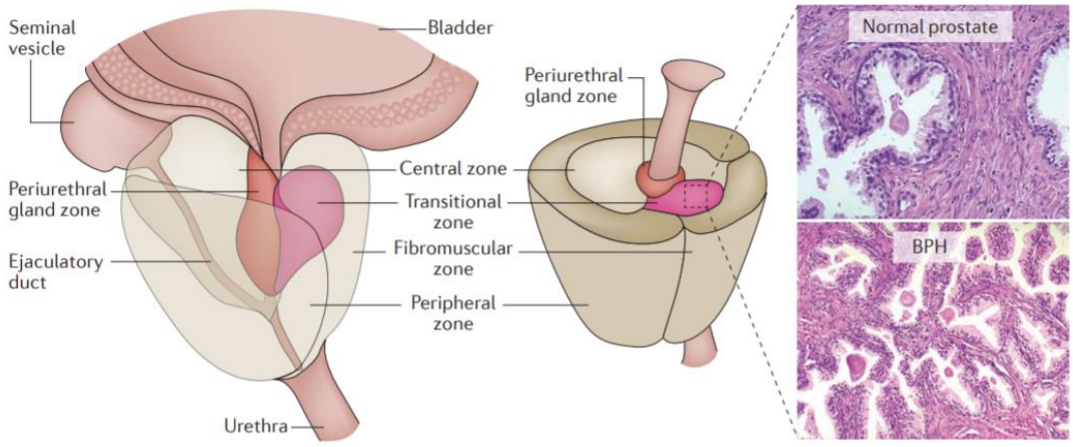

Figure 1. Anatomical regions of the prostate gland. Schematic representation of the zonal anatomy of the prostate as proposed by McNeal (left). Histological slides of a normal prostate and BPH (right). Image adapted form [6].

The mature prostate epithelium is comprised of acini and ducts lined by several types of cells (**Figure 2**) with different morphology and functions [1]. The luminal acinar cells are long columnar epithelial cells that secrete glandular fluid involved in semen production. These cells are terminally differentiated and express characteristic markers such as cytokeratin 8 and 18, prostate specific antigen (PSA) and high levels of the androgen receptor (AR), as they have an androgen-dependent growth [7,8].

The basal cells are non-secretory cells located between the luminal layer and the basement membrane, which allows the separation between the lumen and the stroma and are thought to protect luminal cells from oncogenic insults. They express markers such as cytokeratin 5 and 14, and p63 [9]. Notably, basal cells are proliferating cells that express low or undetectable levels of the AR and PSA compared to luminal cells, as their growth is not directly controlled by androgens [7]. It is believed that prostate stem cells reside in the basal cell layer [10]. Within the basal layer, "intermediate cells" that express luminal and basal markers can be found however, weather these cells represent a functionally distinct cell type remains unknown [8].

Finally, sparsely neuroendocrine cells can be basally found secreting neuropeptides and other hormones, which are thought to support the growth and differentiation of luminal cells by paracrine and endocrine signalling [8,11].

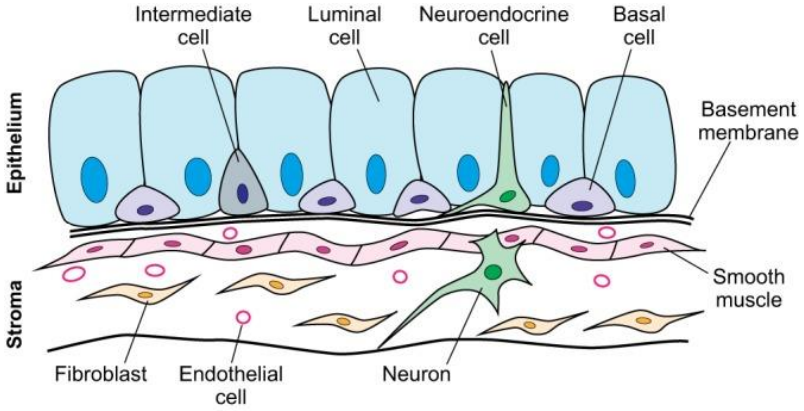

Figure 2. Prostate epithelium differentiated cells. The adult prostatic epithelium is composed of basal cells that line the basement membrane, luminal cells and disperse neuroendocrine and intermediate cell populations. This epithelium is arranged in ducts that are adjacent to the stromal compartment formed by fibroblasts, smooth muscle and neuronal and vascular components. Image adapted from [8].

1.1.2. The role of androgens and the AR in prostate development

The prostatic tissue is fully dependent on androgens and on the androgen receptor for its development and homeostasis. Evidence of this is the natural history of the organ. During childhood, the prostate is small, with a weight of around 2 grams. During puberty, when serum androgens reach adult levels, the gland undergoes an exponential growth, increasing to around 20 grams. After this period, the organ gets to an equilibrium between cell death and proliferation, regulated by AR signalling, and its weight stabilizes [12]

The AR is a member of the nuclear receptor superfamily that is activated and translocated to the nucleus upon ligand interaction [13]. The AR along with androgens are essential for the development of the prostate gland *in utero*. It is confirmed by the absence of prostatic embryonic structures (or prostatic buds) in males that have spontaneous AR mutations that reduce the affinity for its ligands [14,15]. In the adult prostate, the AR continues to be critical for the maintenance of the organ. Depletion of androgens in adult males causes prostatic shrinkage due to the loss of secretory luminal cells. Nonetheless, the gland is able to recover its normal size after restoration of physiological androgen levels [16].

The testicles are the primary source of androgens, which secrete mainly testosterone that is converted into its more active metabolite DHT in the prostate, by 5α-reductase enzyme [17]. It has been shown that individuals with non-functional forms of this enzyme and consequently, reduced levels of DHT, present small prostates with ambiguous genitalia. However, treatment with DHT during adulthood is able to increase prostate size [18].

All these represent clear evidence of the crucial role that the AR and androgens play during prostate development and proper function of the adult gland. However, a better understanding of the underlying mechanisms of these physiological processes may reveal important notions about their functions during the development of prostatic diseases, such as prostate cancer.

1.2. Prostate cancer

1.2.1 Prostate cancer epidemiology

Prostate cancer (PCa) is the second most common type of cancer and the fifth leading cause of cancer-related deaths among men (**Figure 3**). Almost 1.4 million of new cases and more than 375000 deaths were reported worldwide in 2020 [19].

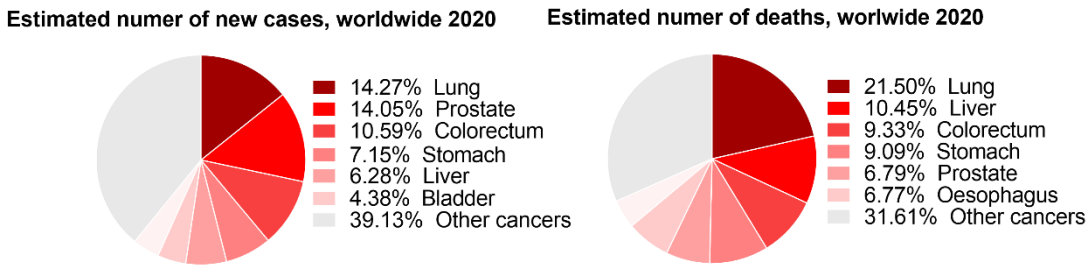

Figure 3. Estimated new cases and deaths of the most common types of cancer worldwide in 2020. The area of the pie chart reflects the proportion of the numer of cases (left) and deaths (right) for each cancer type. Data extracted from [19].

PCa is rarely diagnosed in men younger than 45. However, incidence raises from this age until 70, being 66 the average age at the time of diagnosis [20]. PCa incidence is highly variable worldwide, being northern Europe, North America and Australia/New Zealand the regions with the highest incidence, followed by Eastern Europe, South America, southern Africa and Western Asia. The lowest incidence is found in southern and eastern Asia and the rest of Africa [21]. The reasons for this worldwide incidence variation are not clear but, in some studies, it has been attributed to the extensive PSA testing performed in USA and Europe and the disparities in diagnostic practices across countries [22,23]. Differences upon racial and ethnic groups have also been reported. African American men display the highest incidence worldwide and are also more likely to develop the disease earlier in life. Moreover, European black men and Caribbeans also show similar rates. All this suggests that they might share a genetic background that enhances their susceptibility to develop this type of cancer [24].

Although PCa incidence is high, the 5-year survival rate is 98% for all stages combined. However, this rate varies substantially depending on the tumor stage at the time of diagnosis. For patients diagnosed at localised and regional stages (78%), the survival rate is around 100%. Unfortunately, the rate plummets below 30% for those patients that are diagnosed with metastatic disease (5% of all cases) (**Figure 4**) [20].

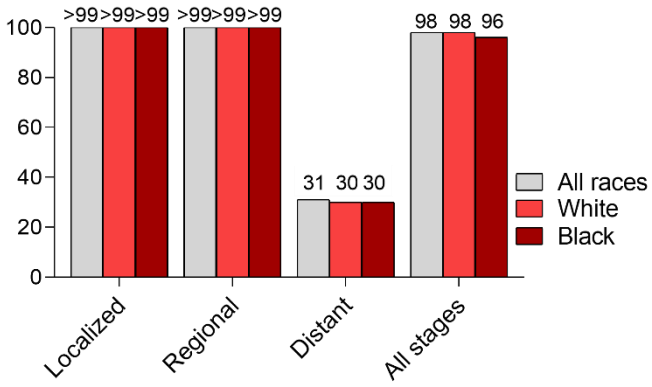

Figure 4. Five-year relative survival rates for prostate cancer by race and stage at diagnosis. Most PCa at the time of diagnosis are fully contained inside de prostate (almost 80% of all cases). Around 15% of cases have loco-regional (local positive lymph nodes) metastasis and a minority (5%) have distant metastasis at diagnosis. The 5-year overall survival of patients with localized disease is almost 100%, whereas that of patients with distant metastases is around 30%. Image adapted from [25,26].

Something similar to what happens with PCa incidence can be observed with mortality. PCa international mortality rates differ substantially worldwide. It rises with age, and the disparities are more evident in men younger than 75. Statistics show that mortality in African American men is 39,9/100000 compared with 18.2/100000 in non-Hispanic white men, which represents a 2.2-fold higher rate [27].This suggests that African American men, not only have a genetic background that makes them more susceptible to develop PCa, but also it is associated with more aggressive forms of the disease. All these, along with more limited access to PSA testing and effective treatments, trigger that African American men display the highest PCa mortality rates [28].

1.2.2. Etiology and risk factors

For such a common disease as PCa, little is known about its etiology compared to other common cancers. In part this is due to the experimental difficulties associated with the isolation of the cell of origin that undergoes oncogenic transformation and gives rise to tumor initiation. It is believed that the aggressive potential, treatment response and patient outcome depends, at least in part, on the cell of origin [29]. This is of particular interest in the case of PCa, which is a very heterogeneous disease with widely different outcomes. It can vary from indolent PCa that can be supervised without therapeutic intervention (active surveillance) to exceptionally aggressive tumors that require intense treatment regimens [30].

Although the hypothesis has not been conclusively demonstrated, it is believed that prostatic intraepithelial neoplasia (PIN) is the premalignant precursor lesion of PCa [31]. It is characterised by low number of basal cells, the presence of luminal epithelial hyperplasia

and marked increase of proliferation markers (**Figure 5**). Likewise, prostate adenocarcinomas, which are the most common type of PCa, have deficient basal cells with predominant luminal phenotype [32]. Because of pathological diagnosis based in the absence of basal markers, luminal cells have been traditionally considered the cells of origin of PCa [33,34]. Yet, it has also been demonstrated that basal cells are able to initiate prostate tumors with luminal phenotype in immunodeficient mice by transformation through oncogenic signalling [35]. Other studies have suggested that another type of cells known as "intermediate cells" may also be implicated in prostate carcinogenesis [36]. Despite all the efforts, no study has been able to definitely prove the cell of origin of PCa, most likely because, given the high tumoral heterogeneity, PCa might arise from multiple cell types [37].

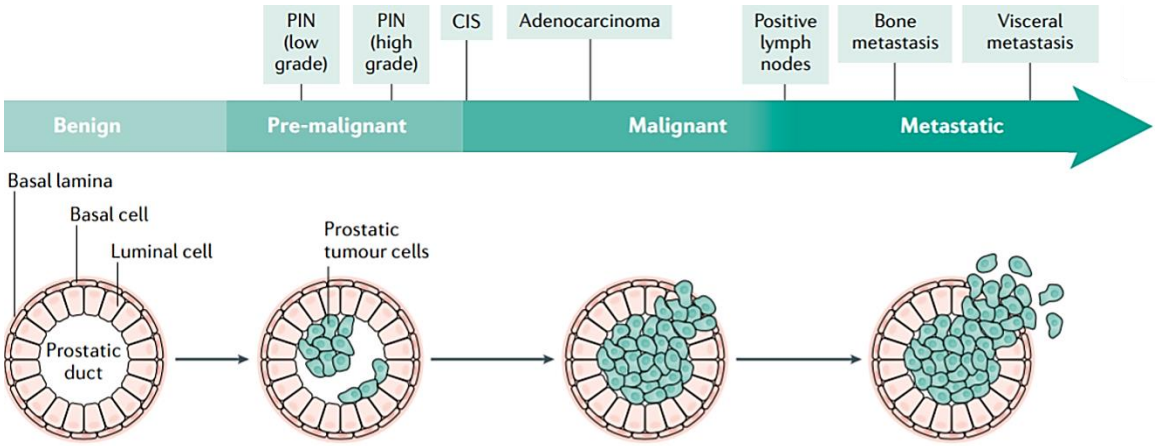

Figure 5. Stages of PCa initiation and progression. PIN, prostatic intraepithelial neoplasia; CIS, carcinoma in situ. Image adapted from [26].

The fraction of PCa that can be related with specific risk factors is very low compared to other types of cancer. The only consistent risk factors identified nowadays are age, race and family history (**Table 1**). The number of senior men diagnosed with PCa is increasing due to higher life expectancy and PSA testing. The age-related risk rises after the age of 50 in white men and after 40 in black men. The disease is very rare in men younger than 40 and the 85% of the cases are diagnosed after the age of 65 [28].

In accordance with twin studies, the heritability of PCa is one of the highest, ranging from 42 to 57% [38]. It is estimated that around 20% of PCa patients have a family history, which seems not only related with a shared genetic background but also to lifestyle habits and environmental factors [28], facts that are supported by migrant studies [39,40]. Given the strong familial component, several family-based studies have been performed to identify causative mutations, but without great success, highlighting the polygenic nature of the disease. Only the presence of germline mutations in certain genes, such as BRCA1/2 and HOXB13 are being used in cancer risk assessment and disease management [41].

Other factors that have demonstrated probable association with PCa are the diet, obesity (in the case of advanced PCa), physical inactivity, infections, chronic inflammation, hyperglycemia and exposure to ionizing radiation and chemicals [42].

Table 1. Factors strongly associated with PCa.

Factor	Estimated RR	Additional information	Refs.
Total PCa			
Age	12,9%	Risk of PCa from age 75	43
Family history	2,0	RR for first-degree relative	44
African descent	1,7	RR compared to non-Hispanic withes	45
Advanced PCa			
Obesity	1.09	RR per 5 Kg/mm^2	46

Abbreviations: RR, relative risk; PCa, prostate cancer; Refs., references

1.2.3. Molecular landscape of prostate cancer

PCa is a highly molecularly and clinically heterogeneous disease [47]. The emergence of next-generation sequencing technologies has allowed the identification of common genomic alterations and susceptible genes which may help to distinguish between different molecular subclasses of PCa with different evolutionary courses and clinical outcomes. However, their application into the clinics is complicated due to the heterogeneous natural history of the disease [48].

As mentioned in previous sections, PIN is considered a preneoplastic lesion which develops towards PCa during a process which may last decades. Furthermore, it has been found that in patient biopsies, high grade PIN (HGPIN) coexist adjacent to PCa areas sharing some molecular alterations [49]. PCa progression is divided into three main stages: localised, metastatic castration sensitive (mCSPC) and castration resistant prostate cancer (CRPC) each of which is characterised by different genomic alterations (**Figure 6**).

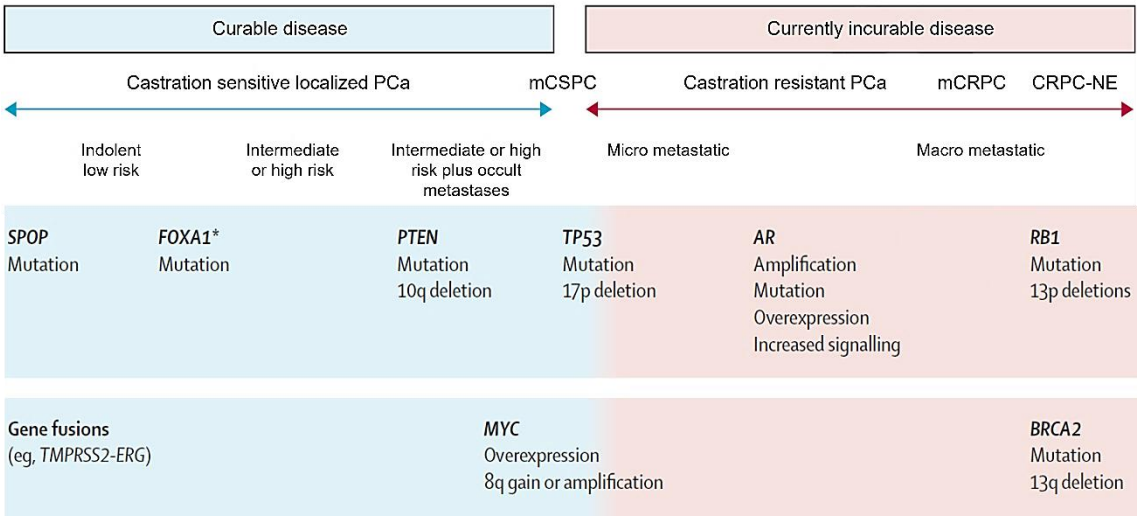

Figure 6. Most common mutations in PCa according to their enrichment at different stages of the disease. PCa is a very heterogeneous disease and patients usually harbour complicated combinations of several genetic alterations. TMPRSS2–ERG fusion, PTEN and RB1 deletion, TP53 mutation and amplification of MYC are very common genetic changes found at all stages of the disease. SPOP mutations are enriched in CSPC, and AR alterations are common in CRPC. Image adapted from [50]. PCa, prostate cancer; mCSPC, metastatic castration sensitive prostate cancer; mCRPC, metastatic castration resistant prostate cancer; CRPC-NE, neuroendocrine castration resistant prostate cancer.

One of the earliest alterations in PCa are gene fusions affecting androgen responsive promoters and members of the E26 transformation-specific (ETS) transcription factor family members. The most common are TMPRSS2-ERG fusions, detected in 50% of patients [51]. Another typical alteration that occurs at early stages of tumor development are SPOP loss of function mutations in 5-15% of tumors and gain of function mutations in FOXA1 gene in 3-5% of cases. Single nucleotide polymorphisms are rare in early PCa, neither are common mutations in the AR gene [52].

In the case of advanced disease, TP53 missense mutations, truncations and deletions are observed in 50% of patients. PTEN alterations, mostly deletions, are found in 40% of cases, whereas in early PCa, alterations in these genes are only found in 10-20% of primary tumors. Furthermore, co-mutation of both genes is very rare in localised tumors (<2%), while it is highly prevalent in metastatic CRPC (mCRPC) (23-33%) [53].

Alterations in the AR that result in increased AR signalling, such as amplifications, gain of function mutations and AR splice variants with ligand-independent activation, are clear drivers of the development of CRPC and are observed in 60-70% of patients [50]. In rare occasions (<2% of all PCa), a total loss of dependency on AR signalling eventually occurs, reaching a stage known as neuroendocrine PCa, which is highly aggressive and treatment refractory [54].

Other frequent alterations characteristic of advanced PCa are gain of function mutations in PIK3CA, PIK3CB or AKT1 in 5% of cases. MYC oncogene overexpression and aberrant activation of Wnt signalling pathway are also common, detected in 20-30% of patients [55]. Around 12% of cases of CRPC harbour Rb1 gene loss, which is highly associated with poor clinical outcome [56].

Genetic aberrations in DNA damage response (DDR) genes also play an important role in PCa. The most common germline mutated genes in PCa are BRCA1, BRCA2, ATM, CHEK2, RAD51D and PALB2, and represent about 12% of mCRPC cases [57]. Moreover, it is also common to find somatic mutations in DDR and mismatch repair genes (i.e. CDK12), occurring in approximately 23% of cases [58]. The high incidence of these mutations has led to development of new targeted therapeutic approaches (e.g., PARP inhibitors) and the recommendation of genetic testing, if possible, for men with metastatic PCa, independently of their family history [41,57].

1.2.4. Diagnosis and staging

1.2.4.1. Diagnosis approximations

At early stages of the disease, PCa is usually asymptomatic. The first signs appear when the tumor grows and exerts pressure on the urethra, obstructing urine flow and causing urinary symptoms. Also, bone pain in hips, ribs or back can be symptoms of already seeded metastases. However, these symptoms are very unspecific and usually related with benign prostatic conditions [59]. Traditionally, PCa has been diagnosed by digital rectal examination (DRE), determination of serum prostate-specific antigen (PSA) levels, followed by a definitive diagnosis by transrectal ultrasound (TRUS) guided biopsy.

Patients that suffer from some of the abovementioned symptoms are subjected to **DRE**. With this technique, asymmetry, abnormalities, or suspicious nodules can be detected in the peripheral zone of the prostate by a specialised urologist. Although its ability for initial detection of PCa is limited to big palpable tumors, an abnormal DRE remains an indication for prostate biopsy, irrespective of the level of PSA, and uncovers around 20% of all prostate cancers [60]. Elevated **serum PSA levels** are strongly related with increased risk of PCa and occurs in 80% of detected cases. Although PSA is an organ specific marker, it is not tumor specific and is not able to discern between benign disorders, such as BPH or prostatitis, and PCa [61]. PSA levels alone only correctly identify PCa in 25 to 30% of cases, and this is why PSA screenings remains controversial, due to risk of overdiagnosis, overtreatment, complications from unnecessary biopsies and debated survival benefit [62]. However, PSA serum levels above 4 ηg/ml are established as a cut-off for biopsy recommendation [63]. The

gold standard for a definitive PCa diagnosis after elevated PSA levels or dubious DRE is the **ultrasound (US)-guided prostate needle biopsy,** which can be performed either by transrectal or transperineal approach [64]. As several studies have associated the transrectal approximation with higher risk of infections, its used is recommended to be abandoned in favour of the transperineal approach [65,66]. The most common approximation consists in obtaining two samples from three areas (base, mid-gland, and apex) on both sides of the peripheral zone, known as 12 core sextant biopsy, in order to better identify the exact location and magnitude of the tumor [67]. If the first biopsy is negative but PSA levels continue high or raising, it is recommended to perform another set of biopsies with extended sample size [68]. However, as some lesions are located in difficult to reach regions or are small, this non-targeted technique can lead to sampling error, missing an aggressive cancer or over-diagnosing an indolent one. For these reasons, efforts are being focused on the development of new strategies to improve detection [64]**. Multiparametric-magnetic resonance imaging (mp-MRI)** have emerged as a useful tool to improve US-guided biopsy, resulting in higher detection rates and improved confident in biopsy results [69]. Specially, software-assisted ultrasound-magnetic resonance imaging (US-MRI) fusion targeted biopsy is becoming a popular method for prostate targeted biopsy. With this technology, the superior lesion detection of MR imaging can be overlapped with the real-time ability of ultrasound guidance, allowing the urologist to target lesions identified by mp-MR imaging [67].

1.2.4.2. Staging

Once the biopsy is analysed and the pathology is established, PCa is graded and staged for patient risk stratification and decision making. The main objective of tumor classification is to group patients with similar outcome, allowing the management of clinical trials with homogenous patient cohorts, the comparison of clinical data from hospitals worldwide and, most importantly, the establishment of treatment recommendations for each group [70].

Gleason score

The Gleason grading system was developed in 1960s by Dr. Donald F. Gleason for PCa pathological description [71]. Since then, it has been the most used and reliable predictor of prognosis of PCa patients. It is based on the histological architecture of the tumor instead of on the individual cellular characteristics that define other cancers. The grade is obtained assigning values from 1 to 5, being 1 the most differentiated glandular pattern and 5 when no glandular architecture remains. Thus, higher numbers correspond with poorly differentiated tumors, which are usually more aggressive and display poor prognosis [72].

Furthermore, the Gleason Score (GS) usually comprises two grades. The first number represents the grade of the most extensive histology, followed by the second most common pattern, if two are present. In the case of three patterns, the GS is composed by the most common followed by the highest, independently of its extent [73].

The Gleason grading system has undergone several adjustments since its creation. In 2014, the International Society of Urological Pathology (ISUP) introduced extensive modifications in order to provide more accurate patient stratification and reduce overtreatment of indolent PCa. The number of grading categories were reduced, and the original Gleason Scores were organised into "Grade Groups" 1 to 5 (**Figure 7**) [74].

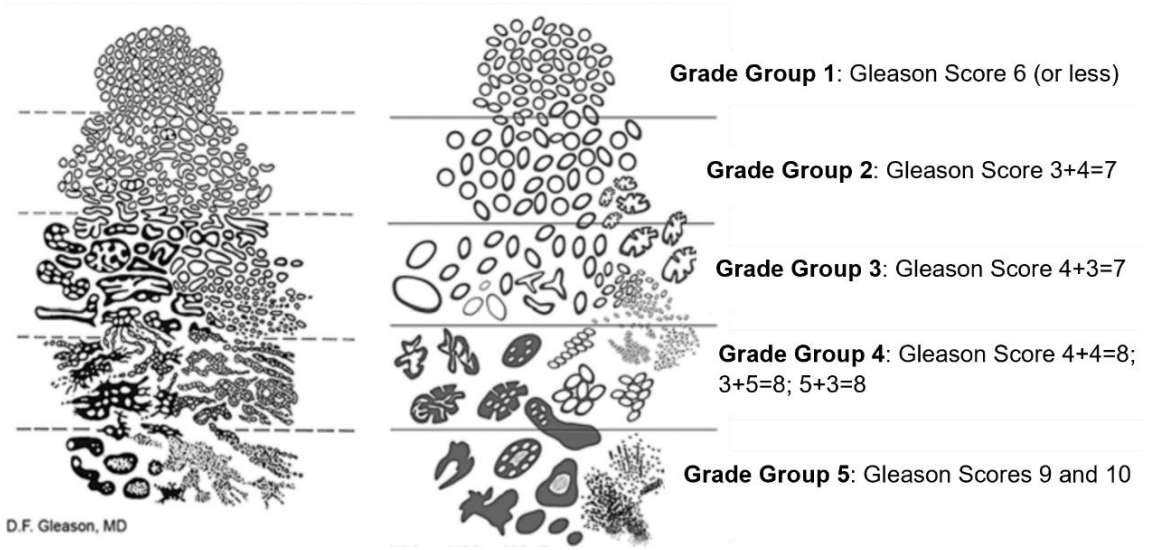

Figure 7. PCa histologic patterns. Original (left) and 2014 modified ISUP Gleason schematic diagrams (right). In either case, increasing number means increased severity of the disease. Image adapted from [74]

Clinical Tumour Node Metastasis (TNM) classification of PCa

Apart from the Gleason Score, PCa staging is completed following the TNM classification. It is an anatomically based system that takes into account the size and extent of the tumor (T), the affection of lymph nodes (N), and the presence of distant metastases (M) (**Table 2**). The Union for International Cancer Control (UICC) TNM classification is internationally accepted and has been the standard for cancer staging for over 50 years, being its 8th edition the most recently published [75].

Table 2. Tumor node metastasis (TNM) classification of PCa. Table adapted from [75].

T - Primary Tumour (stage based on DRE)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour that is not palpable
	T1a Tumour incidental histological finding in 5% or less of tissue resected
	T1b Tumour incidental histological finding in more than 5% of tissue resected
	T1c Tumour identified by needle biopsy (e.g., because of elevated prostate-specific antigen [PSA])
T2	Tumour that is palpable and confined within the prostate
	T2a Tumour involves one half of one lobe or less
	T2b Tumour involves more than half of one lobe, but not both lobes
	T2c Tumour involves both lobes
T3	Tumour extends through the prostatic capsule
	T3a Extracapsular extension (unilateral or bilateral)
	T3b Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall
N - Regional (pelvic) Lymph Node Metastasis*	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M - Distant Metastasis**	
M0	No distant metastasis
M1	Distant metastasis
	M1a Non-regional lymph node(s)
	M1b Bone(s)
	M1c Other site(s)

Abbreviations: DRE, digital rectal examination
* Metastasis no bigger than 0,2 can be designated pNmi
** The most advanced category is used when more than one site of metastasis is present. (p)M1c is the most advanced category

The recommendation of the European Association of Urology (EAU) to obtain precise patient stratification is to use the TNM classification in combination with the ISUP 2014 system for the grading of PCa. Furthermore, these data in combination with PSA levels are used to classify patients with localised and locally advanced PCa into risk groups for biochemical recurrence (BCR) (**Table 3**) [70].

Table 3. EAU risk groups for BCR of localised and locally advanced PCa. Table adapted from EAU Guidelines [70].

Low-risk	Intermediate-risk	High-risk	High-risk
Localised PCa			Locally advanced PCa
PSA < 10 ŋg/ml	**PSA** 10-20 ŋg /ml	**PSA** > 20 ŋg /ml	Any **PSA**
or **GS** <7 (ISUP Grade 1)	or **GS** 7 (ISUP Grade 2/3)	or **GS** > 7 (ISUP Grade 4/5)	any **GS** (any ISUP Grade)
or **cT1-2a**	or **cT2b**	or **cT2b**	**cT3-4** or **cN+**

Abbreviations: GS, Gleason score; ISUP, International, Society for Urological Pathology; PSA, prostate specific antigen; PCa, prostate cancer

1.2.5. Prostate cancer management and therapeutic intervention

As mentioned earlier, PCa is a highly clinically heterogeneous disease and after diagnosis there is a wide spectrum of courses that range from clinically insignificant to greatly aggressive disease. These, together with the extensive range of treatment options currently available, result in quite complex management of the disease [76]. The key clinico-pathological factors including TNM stage, PSA levels, and histological grade (Gleason Score), along with newly incorporated factors such as imaging findings, molecular signatures and number of positive biopsy cores [77], help physicians with treatment-decision making. The main therapeutic choices consist on active surveillance for indolent disease, single-modality therapies for low/intermediate-risk patients, such as prostatectomy or radiotherapy, and multimodality treatments for higher risk patients [70].

1.2.5.1. Management of localised and locally advanced prostate cancer

Active surveillance

Active surveillance (AS) is an approved option for the initial management of certain patients with localised PCa who have low risk of progression in order to avoid unnecessary treatment. Patients remain under close observation with regular follow-ups consisting on clinical examination, PSA testing, biopsies and mp-MRI [78]. If changes towards a higher risk tumor are detected, interventional treatment is offered.

It is important not to mistake active surveillance with watchful waiting (**Table 4**). It is also an observational approach, but in this case it is thought for elderly, fragile, asymptomatic patients, where the related toxicity associated to treatment does not justify the benefits in survival [79]. The patients are clinically watched for the development of progression and, if this is the case, they are treated with palliative therapies according to their symptoms, to maintain their quality of life [70].

Table 4. Differences between active surveillance and watchful waiting. Table adapted from EAU Guidelines [70].

	Active surveillance	Watchful waiting
Treatment objective	Curative	Palliative
Follow-up	Pre-defined schedule	Patient-specific
Markers	DRE, PSA, mp-MRI, re-biopsy	Dependent on the development of symptoms and progression
Life expectancy	> 10 years	<10 years
Main aim	Minimise overtreatment without compromising survival	Minimise treatment related toxicity
Inclusion criteria	Low-risk patients	Can apply to patients with all stages

Abbreviations: DRE, digital rectal examination; PSA, prostatic specific antigen; mp-MRI, multiparametric magnetic resonance imaging

Radical prostatectomy

Surgery is one of the therapeutic choices to treat patients with low/intermediate-risk localized PCa. The main objective of this approach is the ablation of the tumor while preserving, if possible, urinary continence and erectile function. The intervention implies removing the entire gland with its capsule intact [79] and it can be performed as open surgery, laparoscopic (LRP), or robotic assisted (RARP). During the last years, RARP has been gaining popularity worldwide. Its benefits compared with open surgery are reduced blood loss, shorter hospital stays and less pain. However, the long-term oncological benefits are still unproven [80]. After prostatectomy, PSA levels should be undetectable. A raise in serum PSA > 0,4 ŋg/ml indicates biochemical recurrence and is a predictor of the appearance of metastases [81].

Radiation therapy

Radiotherapy (RT) is a stablished modality for the treatment of low/intermediate PCa patients at local or regional stage [82]. Furthermore, it has been shown that men with high-risk cancer receiving RT plus androgen depravation therapy (ADT) displayed a significant survival benefit compared to ADT alone [83]. External-beam radiation therapy (EBRT) and brachytherapy are two important forms of RT offered to the abovementioned patients. EBRT consists on delivering high-energy X-rays or electron beams towards a patient's tumor. Brachytherapy implies the implantation of radioactive seeds directly into the prostate [79].

The use of this radical local-therapies is related with a detriment of the quality of life, giving place to incontinence, reduced sexual function and radiation toxicity [79]. Minimally invasive ablative therapies for localized PCa are emerging as an alternative to these strategies. Cryotherapy, high intensity focused ultrasound, interstitial laser thermotherapy and

electroporation are some examples, but remain under research. They seem to have less high grade associated complications but so far, long-term oncological results are uncertain [50]. All things considered, in a usually slow growing disease that mainly affects elderly men, it is highly important to consider the balance between control of primary disease and treatment associated comorbidities.

1.2.5.2. Management of advanced or metastatic prostate cancer

After first line treatment, within a period of 10 years, 20-40% of patients treated with RP and 30-50% treated with radiotherapy will suffer BCR, characterized by a rise in PSA after prostate-directed therapy, but without evident metastasis. [84]. 5 years after BCR around 37% of patients will develop metastatic disease known as metastatic castration sensitive prostate cancer. Furthermore, almost 6% of men display distant metastasis at initial diagnosis [85,86]. PCa progression is a continuum characterized by the presence or absence of metastasis and the sensitivity or resistance to ADT. The wide range of therapeutic options that continue rapidly increasing, make difficult to apply results from clinical trials, patient management and treatment choice [87]. **Figure 8** represents a summary of the current treatment options available for each stage of the disease.

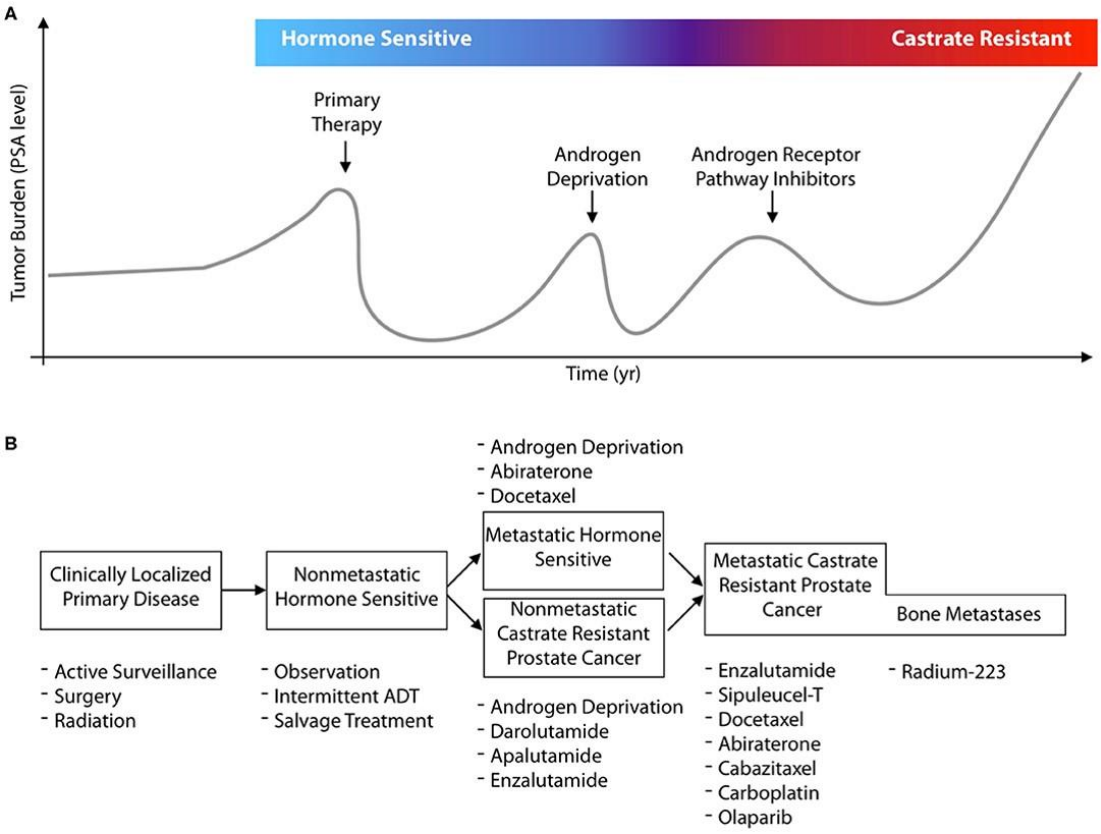

Figure 8. Schematic representation of the natural development of PCa and the most common therapies for each stage of the disease. Image from [88].

Hormonal therapy

As PCa is described as a hormone-dependent tumor, the AR and its pathway have been considered an important target for the development of effective therapies [89]. Androgen deprivation therapy remains the first line treatment for advanced PCa, and its main objective is to reduced androgen circulating levels and androgen receptor signaling [90]. Surgical castration, known as orchiectomy, is the original form of ADT, and remains in use worldwide. However, the psychological trauma to the patients and the irreversibility of the procedure is leading to its replacement by chemical approaches [89].

As the hormonal control of testosterone production is initiated in the hypothalamus by the production of luteinizing hormone releasing hormone (LHRH), LHRH agonist and antagonists that target the LHRH receptor were among the first chemical approaches to reduce androgen signaling [91]. With these compounds, chemical testosterone castration levels are achieved (<50 ng/dL) [70], avoiding the physical and psychological distress caused by orchiectomy.

Antiandrogens are also commonly used drugs for the treatment of advanced PCa. Flutamide, nilutamide and bicalutamide are some of the first that were developed and

introduced into the clinical practice and are recognized as first-generation antiandrogens [70]. These compounds act on the androgen receptor, avoiding the binding with its ligands. They do not reduce testosterone circulating levels, and for this, libido and bone mineral density are commonly not affected, unlike as it happens with LHRH agonist/antagonists [92]. However, they are usually administered in combination with LHRH agonist or antagonist, to accomplish complete androgen blockade, especially in men with metastatic PCa [93].

Once the treatment with drugs that target the LHRH receptor or with first-generation antiandrogens is started, it is only a matter of time that CRPC develops. Since most CRPC cases remain AR-dependent through several mechanisms [94], (that will be further explained in section 1.2.6), second-generation compounds known as androgen receptor pathway targeting agents (ARTA), with wider mechanisms of action, have emerged**. Abiraterone acetate** (ZYTIGA®) suppresses intracellular androgen biosynthesis at the adrenal gland, testes and inside cancer cells by inhibiting the enzyme CYP17A1 [95]. In 2011 it was approved for patients with mCRPC and in 2018 the indication was expanded for mCSPC [96,97]. Furthermore, it has been recently demonstrated that the reduction of testosterone to sub-castration levels by the combination of abiraterone plus ADT can bring additional benefits to PCa patients. This points out that low androgen levels after castration can still stimulate PCa growth [98]. **Enzalutamide** (XTANDI®) and **Apalutamide** (ERLEADA™) are non-steroidal second-generation antiandrogens with stronger binding affinity for the AR than the first-generation ones. They act by binding to the AR into the androgen binding site, inhibiting AR nuclear translocation and AR-DNA interaction [99 100]. Enzalutamide was the first second-generation antiandrogen approved by the Food and Drug Administration (FDA), for the treatment of metastatic (2012) and non-metastatic CRPC (nmCRPC) (2018). More recently it has also been approved for the treatment of mCSPC (2019) [101]. Apalutamide received FDA approval in 2018 for the treatment of nmCRPC [102] and in 2019 for mCSPC [103]. **Darolutamide** (Nubeqa®) [104] is the most recently approved second-generation antiandrogen, in 2019, for the treatment of nmCRPC [105]. It acts similarly to enzalutamide and apalutamide, as an antagonist of the AR, but with some additional characteristics. It can target some AR mutants that cause resistance to other antiandrogens and also, its different chemical characteristics avoid it from crossing the blood brain barrier, suggesting a lower seizure risk, which is a common adverse event observed in enzalutamide treated patients [106]. Similar as it happens with abiraterone, improved outcome is expected upon the combination of ADT with these new compounds, which is now under investigation with promising initial results. **Figure 5** shows the main and most recent clinical trials involving second-generation antiandrogens for the treatment of advanced PCa.

Table 5. Main clinical trials with second-generation antiandrogens for the treatment of advanced PCa performed since 2017. Table adapted from [107].

Agent	Clinical trial	Patient number	Primary endpoint results (intervention *vs.* control)			
			Primary end point	Int.	Control	HR (95% CI, P)
Metastatic castration sensitive prostate cancer (mCSPC)						
Abiraterone	LATITUDE NTC01715285	Abiraterone + prednisone + ADT (597) *vs.* ADT (602)	Median OS	NR	34.7 months	0.62; 95% CI 0.51–0.76, P <0.001
			Median rPFS	33 months	14.8 months	0.47, 95% CI 0.39–0.55. P <0.001
	STAMPEDE NCT00268476	Abiraterone + prednisone+ ADT (957) *vs.* ADT (960)	OS (3 years)	83%	76%	0.63, 95% CI 0.52–0.76, P <0.001
			Failure-free survival (3 years)	75%	45%	0.29, 95% CI 0.25–0.34, P <0.001
Enzalutamide	ENZAMET NCT02446405	Testosterone suppression +enzalutamide (563) *vs.* testosterone + FG-AA	OS (3 years)	80%	72%	0.67, 95% CI 0.52–0.86, P = 0.002 (34 months follow-up)
	ARCHES NCT02677896	ADT + enzalutamide (574) *vs.* ADT + placebo	rPFS (median)	NR	19 months	0.39, 95% CI 0.30–0.50, P <0.001
Apalutamide	TITAN NCT02489318	ADT + apalutamide (525) *vs.* ADT + placebo (527)	OS (24 months)	82.4%	73.5%	0.67, 95% CI 0.51–0.89, P = 0.005
			rPFS (24 months)	68.2%	47.5%	0.48, 95% CI 0.39–0.60, P <0.001
Non-metastatic castration resistant prostate cancer (nmCRPC)						
Apalutamide	SPARTAN NCT01946204	ADT + apalutamide (806) *vs.* ADT + placebo (468)	MFS (median)	40.5 months	16.2 months	0.28, 95% CI 0.23–0.35, P <0.001
Enzalutamide	PROSPER NCT02003924	ADT + enzalutamide (933) *vs.* ADT + placebo (468)	MFS (median)	36.6 months	14.7 months	0.29, 95% CI 0.24–0.35, P <0.001
Darolutamide	ARAMIS NCT02200614	ADT + darolutamide (955) *vs.* ADT + Placebo (554)	MFS (median)	40.4 months	18.4 months	0.41, 95% CI 0.34–0.50, P <0.001

Abbreviations: Int., intervention; ADT, androgen deprivation therapy; MFS, metastasis-free survival; NR, not reported; OS, overall survival; rPFS, radiographic progression-free survival

Chemotherapy

Mitoxantrone, a type II topoisomerase inhibitor, was the first cytotoxic chemotherapy approved for the treatment of mCRPC after progression on ADT. It was used as a pain palliative, as no survival benefits were observed and it is no longer commonly used [108]. **Docetaxel**, a taxane that prevents microtubule depolymerization, was the first systemic therapy to demonstrate survival benefit on mCRPC patients and in 2004 was stablished as the standard of care for these patients [109]. **Cabazitaxel** is a second-generation taxane. It has a similar mechanism of action than docetaxel but exhibits less affinity for P-glycoprotein (P-gp) efflux pumps, which have been related with docetaxel-resistance [110]. It was approved in 2010 for the treatment of docetaxel-refractory mCRPC, as it was the first drug to show survival benefits in this set of patients [111]. Platinum-based chemotherapy is not commonly used in PCa. However, some mCRPC patients with DDR gene defects, neuroendocrine characteristics or very aggressive variants that rapidly become AR-independent seem to benefit from this type of treatment [50].

Poly(ADP-ribose) polymerase (PARP) inhibitors

PARP inhibitors have long been used for the treatment of breast and ovarian tumors harboring germline DDR gene defects, especially in those cases affecting BRCA1 and 2 genes. In PCa, alterations in DDR genes such as BRCA1/2, ATM, and CHECK2 among others, are detected in around 23% of mCRPC cases [58]. The genomic instability and other vulnerabilities caused by these alterations generate a weakness on tumor cells that can be exploited with PARP inhibitors, inducing synthetic lethality. A good example of this is olaparib, a PARP inhibitor that demonstrated significant response rates in several clinical trials performed on mCRPC patients with DDR gene alterations [112–114]. These results led to olaparib FDA approval in 2020 for the treatment of this subset of patients who have progressed on standard therapies. Other PARP inhibitors, such as rucaparib, nitaparib, talazoparib and niraparib have shown similar efficacy [50]. On the bases of that discoveries, the National Comprehensive Cancer Network has set the recommendation of germline testing for DDR genes in all patients with advanced or high-risk localized PCa, regardless of family history, in order to improve and facilitate therapy decision making [115].

Immunotherapy

Prostate cancer is known to have a low proportion of neoantigens compared to other solid tumors [116]. Furthermore, the prostate tumor microenvironment has been described to be quite immunosuppressive and to interfere with natural killer cell activity [117]. Altogether, this evidence suggests that PCa tumors are less likely to respond to immunotherapy. However,

based on the benefits observed in other tumors, immunotherapy is also under consideration for PCa treatment. Immune checkpoint inhibitors, such as monoclonal antibodies against CTLA-4, programmed death-1 (PD-1) or its ligand PD-L1, have changed the clinical practice in many cancers. Still, they have failed to demonstrate strong efficacy in PCa [118]. Recently, **pembrobolizumab**, an anti-PD-1 antibody, exhibited an objective response of 50% in a subset of patients harboring microsatellite instability (MSI) caused by a dysregulated mismatch repair (MMR) system [119]. Similarly, a subset of CRPC patients with alterations in CDK12 were recently reported to perform improved response to immunotherapy, which was associated with an increased neoantigen burden due to higher genomic instability [120]. Anyhow, based on these results, in 2017 the FDA approved the use of pembrolizumab for the treatment of mCRPC patients with MMR defects and/or MSI [121]. **Sipuleucel-T** is a cellular immunotherapy that contains autologous antigen-presenting cells (APC). These APC are activated by being exposed to prostatic acid phosphatase (PAP), a tissue antigen expressed by cancer cells, linked to granulocyte-macrophage colony-stimulating factor, prior to their reinfusion into the patient [122]. It was approved by the FDA in 2010 for the treatment of asymptomatic or minimally symptomatic mCRPC, becoming the first approved vaccine for the therapy of solid tumors [123]. However, only mild, long-term clinical benefits have been reported and the high costs and complex procedures limit its used nowadays [124].

Bone-targeted therapy

The bones are the most common site of metastasis in mCRPC patients. Approximately 90% of men who develop metastasis are diagnosed with bone lesions that are responsible of important morbidity [125]. Radium-223 is an alpha-emitting radionucleotide that mimic calcium. It is taken up by osteoblastic bone metastasis exerting a cytotoxic effect on tumor cells and disease-promoting osteoblast, avoiding pathologic bone formation [126]. In several clinical trials, Radium-223 has shown significant prolonged survival, quality of life benefits and delayed onset of skeletal-related events, which have led to its indication for mCRPC patients [127,128].

Signaling pathway inhibitors

PTEN is a well described key tumor suppressor gene that regulates PI3K-AKT-mTOR pathway. PTEN loss is a very common alteration in mCRPC (50% of cases) which leads to PI3K-AKT pathway activation and has been related to abiraterone and enzalutamide resistance [129]. Preclinical studies have shown that PTEN deletion can recapitulate all the stages of the disease, including the development of metastasis [130]. Furthermore, reciprocal feed-back mechanisms between PI3K-AKT-mTOR and AR pathways have been described. In line with this, the dual targeting of both pathways was shown to significantly inhibit tumor

growth in PTEN-null enzalutamide-resistant *in vivo* models [131]. However, results from the first clinical studies with some AKT inhibitors only performed limited efficacy [132]. The AKT inhibitor named ipatasertib has reached phase 3 clinical development for mCRPC patients and combination with abiraterone is currently being evaluated. Preliminary results showed improved outcomes in mCRPC patients with PTEN-loss compared to those without this alteration, pointing out the importance of patient stratification [133].

Stimulating mutations of the Wnt-β-catenin pathway might also play an important role in advanced PCa. They are quite rare at initial stages of the disease, but the signaling pathway is usually overactivated at late stages [134]. In fact, activating mutations in β-catenin have been related with worse overall survival and enzalutamide resistance [135]. Novel compound interfering with Wnt signaling pathway have reached clinical phases, but no definitive efficacy results are available yet [136].

1.2.6. Prostate cancer progression to CRPC

As aforementioned, ADT is the standard of care for those patients who recur after locoregional treatments. The rationale of that is based on the theory of Huggins and Hodges, who demonstrated in 1941 that androgens and the androgen receptor are essential for the growth and survival of PCa cells [137,138]. Despite an initial response of around 80 to 90% of cases achieving PSA decline, almost all patients will become refractory after 2-3 years, reaching a state known as CRPC, which is highly aggressive and a lethal form of the disease [139]. CRPC is defined as an increased in PSA and/or radiographic progression despite castrate testosterone levels [140]. There are multiple mechanisms related with the progression of CRPC, and most of them are associated with the androgen receptor and sustained androgen signalling, fact supported by the survival benefit observed in CRPC patients upon treatment with the antiandrogens abiraterone and enzalutamide after ADT progression [141].

The AR is a ligand-dependent transcription factor that belongs to the steroid hormone nuclear receptor superfamily. It is formed by four main functional domains: the N-terminal domain, essential for transcriptional activity; the DNA binding domain (DBD); the hinge region, and the ligand-binding domain (LBD). In the absence of androgens, the AR is located in the cytoplasm, associated with several heat shock proteins (HSP) and other chaperones [142]. Upon ligand binding, the AR suffers a conformational change, dissociates from the chaperone complex, and dimerizes. Then, the AR is translocated into the nucleus where it mediates the transcription of its target genes through the interaction with the androgen response elements (ARE), located in promoter or enhancer regions of the target

genes [143]. The most commonly described mechanisms related with the development of CRPC imply an enhanced activation of the AR and its signalling pathway, as a result of gain-of-function mutations, increased AR expression, intracrine androgen biosynthesis and the emergence of AR splice variants (**Figure 9**). Alternatively, there are cases in which CRPC might be driven by AR-independent mechanisms [26].

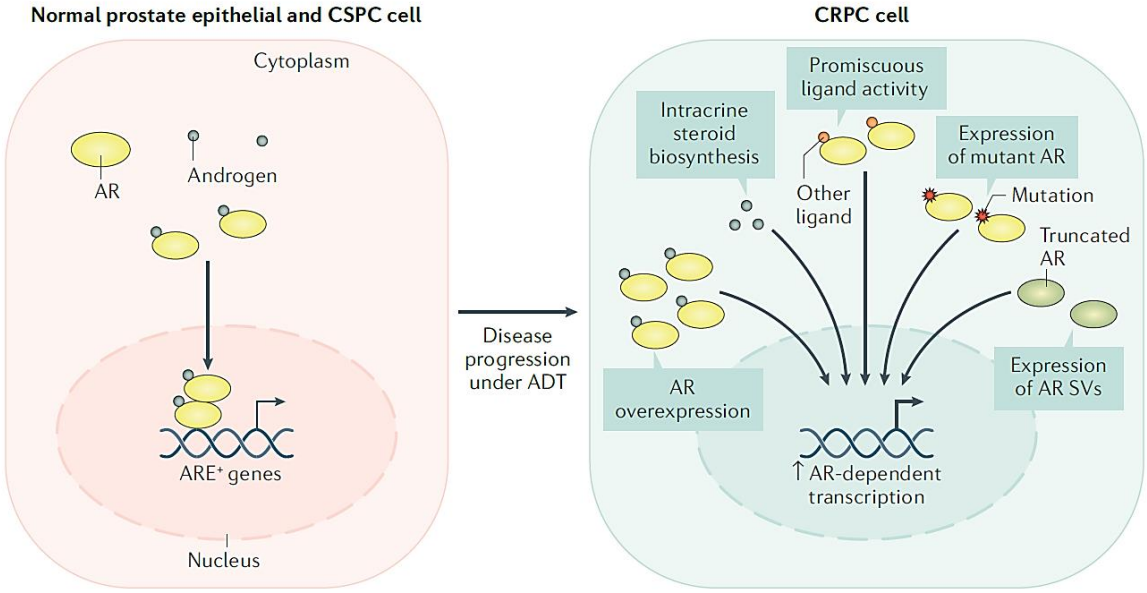

Figure 9. AR-dependent mechanisms involved in the progression of CRPC. PCa progression to CRPC is usually associated with ADT and strongly correlated with alterations affecting the AR or its signalling pathway. The most commonly observed alterations are AR amplification or overexpression, point mutations and AR splice variants that result in constitutively active forms of the receptor, activation of AR signalling by non-specific ligands (promiscuous activity) and the acquired capacity of tumoral cells to synthesize androgens from steroid precursors that leads to AR activation. Image from [26].

Increased AR expression

Around 80% of patients who progressed on ADT display increased AR expression, which can be due to several mechanisms. AR amplification (Xq11-q13 region) is one of the most common alterations detected in around 20-30% of CRPC patients, though rarely found in untreated PCa [139]. Initially, it was believed that AR gene amplification only occurs during castration resistance. However, more recently, it has been found that certain cell subpopulations within hormone-naïve tumors can present this alteration and it is related with worse cancer-specific survival [144]. Alternatively, increased AR levels can be a consequence of higher protein stability generated by certain post-translational modifications or by aberrant interaction with HSP [139]. In all cases, the increased levels of the AR result in higher sensitivity for androgens, allowing PCa growth despite ADT [145].

AR mutations

AR gain-of-function mutations are found in around 50% of CRPC patients, particularly in those that have followed prolonged hormonal therapy, but are very rare in hormone-sensitive patients [58]. There are more than 100 point-mutations described affecting the AR, and the majority of them are located at the LBD. One of the most commonly found mutation is T878A. It was described to extend the AR ligand affinity to other hormones such as estrogens and progesterone, as well as turning antiandrogen agents into agonists that would cause AR activation upon binding [141]. Similar effects have been reported for other mutations such as F876L, H875Y and W742C. All of them have been related with PCa progression, as they are responsible for a continuous activation of the AR, even under castrate levels of androgens and have been associated with resistance to antiandrogens [146,147]. Quite recently, it has been described that these mutations can be easily detected in plasma as cell-free DNA. This could be very useful for the development of biomarkers in terms of individual treatment selection and to improve patient clinical outcome [148].

AR variants

AR variants (AR-Vs) are aberrant forms of the AR protein that lack the LBD (C-terminal region) but have intact N-terminal domain and DBD, which make them constitutively active, even in the absence of androgens [149]. They commonly originate by alternative splicing but also, they can be the product of proteolytic cleavage and nonsense mutations [150]. During the last decades, around 20 different AR-Vs have been identified, and many of them have been related with development of CRPC and resistance to antiandrogens [151]. ARv567 and AR-V7 are the most clinically relevant variants. Their expression is correlated with poor prognosis and AR-V7 is thought to have predictive value for abiraterone and enzalutamide resistance [152,153]. AR-Vs are considered novel therapeutic targets for the treatment of CRPC patients that have progressed on second-generation antiandrogens, and their early detection could help with treatment choice. In fact, compounds that target AR-Vs by binding to their N-terminal domain (EPI-001) or by enhancing their degradation (niclosamide) are under investigation [154].

Intratumoral steroid hormone synthesis

Intracrine androgen biosynthesis is described as another mechanism that allows PCa growth under androgen depleted conditions. Castration, either chemical or surgical, results in a >90% reduction of circulating androgen levels. However, several studies have demonstrated that intratumoral levels of androgens remain high after ADT [94,155]. This suggests that the AR signalling remains active despite castration, which may result in failure

of the procedure [156]. The production of androgens within tumor cells can occur through (I) the conversion of adrenal steroids, androstenedione and dehydroepiandrosterone, to testosterone and DHT [157], and (II) by intratumoral biosynthesis of androgens from cholesterol [158]. All these findings are supported by the increased expression of the enzymes responsible to catalyse these processes observed in CRPC tissues [159,160].

AR-independent mechanisms

As aforementioned, there are some AR-independent mechanisms that contribute to CRPC development and treatment failure. The upregulation of the glucocorticoid receptor, which belongs to the same class of nuclear steroid receptors as the AR, and has overlapping transcriptomes [161], has been described as a clinically relevant mechanism of the development of CRPC and resistance to ARTA [162]. Something similar is described for the upregulation of the progesterone and estrogen receptor (ER), which also belong to the same family and are structurally equivalent to the AR [163 164]. Moreover, abnormal activation of some important signalling pathways, such as PI3K-Akt-mTOR [165], the Src kinase family, and several growth factor pathways, among others, is detected in an important fraction of CRPC patients [141]. Targeting key effectors from these pathways is thought to provide novel therapeutic strategies for the treatment of CRPC and some agents are now under preclinical and clinical evaluation [166–168].

Theoretical models of CRPC development

Although some studies have linked all these alterations with different stages of PCa progression, the exact moment at which these modifications appear and trigger CRPC is not well defined. However, there are two models that were proposed to explain this process: the adaptation, and the selection model (**Figure 10**). The adaptation model proposes that castration resistance occurs by the acquisition of some of the above-mentioned alterations by androgen-dependent cells in response to the altered hormonal environment generated by ADT. These alterations would allow cells to survive and grow under low androgen levels [169]. On the other hand, the selection model suggests that early PCa consists of an heterogeneous mix of cells, of which a minority display castration resistance characteristics. The initiation of ADT would produce a selective pressure, causing apoptosis of androgen-dependent cells and the selection and growth of the castration resistant pre-existing clones [170]. Although evidence supports the selection model, it is difficult to stablish a definitive and exclusive model [171]. It is thought that traits from both can contribute to the development of CRPC [172].

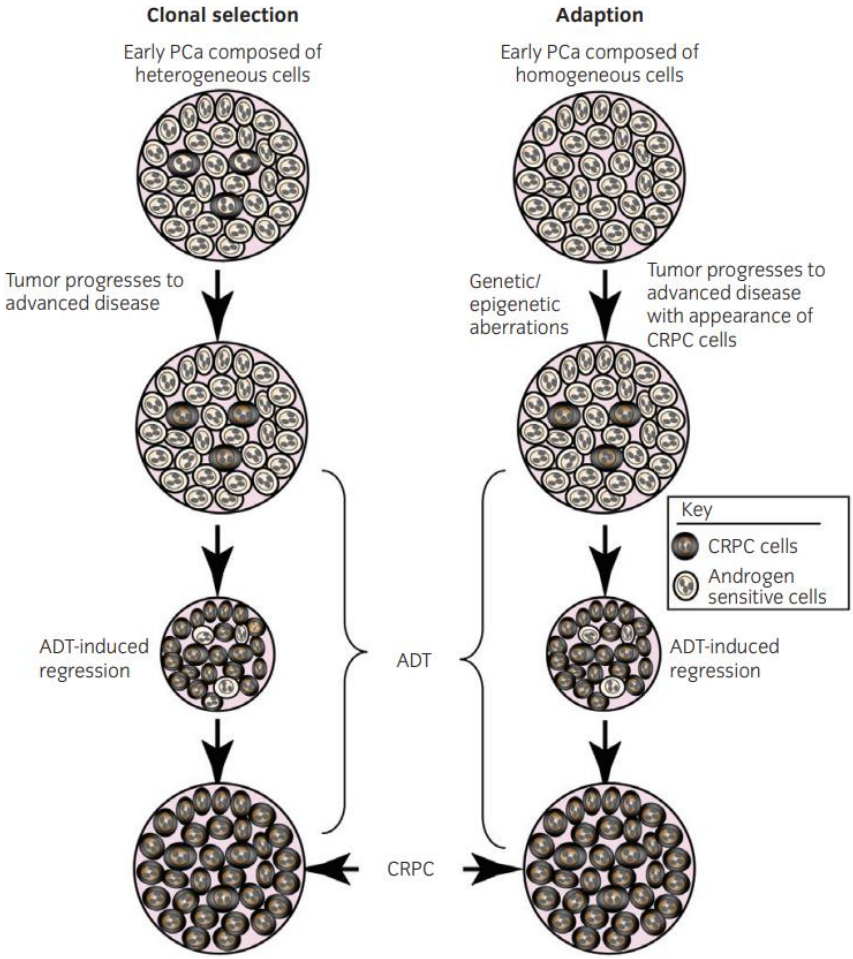

Figure 10. Models of the origin of CRPC. Left scheme shows a representation of the clonal selection model, which postulates that prostate tumors are composed of heterogeneous cells from which a minority are castration resistant. Upon ADT, CPRC cells would be selected, and the androgen dependent ones eliminated. The right scheme represents the adaptation model. In that case, the prostate tumor is proposed to be initially homogeneous, made of androgen dependent cells. The castration resistant clones would appear as an adaptive mechanism to ADT, through genetic and epigenetic mechanisms. Image from [169].

1.3. Targeting mitosis for cancer therapy

1.3.1 Cell cycle: a special focus on mitosis

Cell cycle is a very conserved process regulated by numerous mechanism that ensure correct cell division and faithful transmission of genetic information into the two daughter cells [173]. Cell cycle is divided into five different phases: G0 (gap 0), G1, S (DNA synthesis), G2 and M (mitosis). The four first phases are collectively known as interphase. In G0, cells are in a quiescent, resting state, that can be maintain through their entire lifespan, or enter G1 after stimulation by external growth factors. In S phase, DNA is duplicated and in G2 the fidelity of DNA replication is monitored, and cells are prepared to enter mitosis. Finally, in M phase the genetic material is equally divided, followed by the separation of the cytoplasm (cytokinesis) [174]. Cell cycle progression from one phase to another is strictly controlled. Key regulators are the cyclin-dependent kinases (CDK), a family of serine/threonine kinases. These proteins are ubiquitously expressed during the cell cycle, but its activation is dependent on the fluctuating levels of synthesis and degradation of specific cyclins throughout the different phases [175,176]. Thus, distinct CDK/cyclin complexes are characteristic for each cell cycle phase (**Figure 11**).

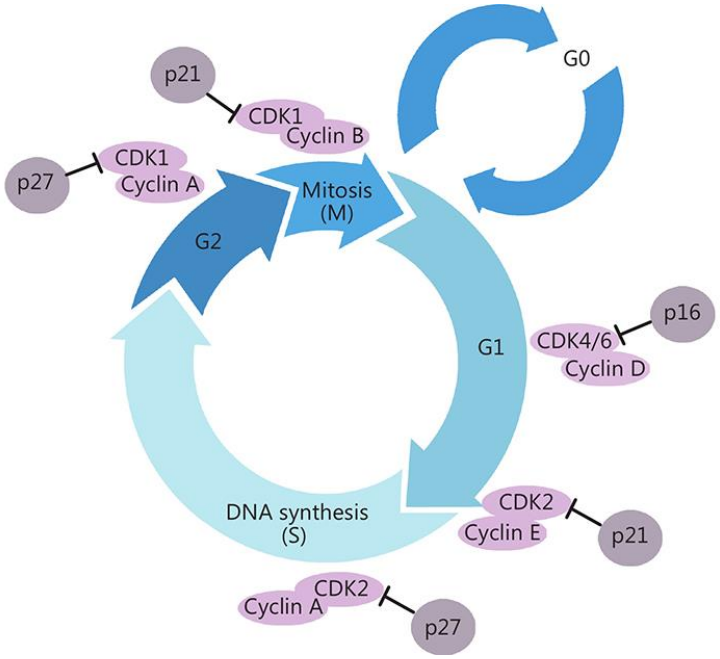

Figure 11. Scheme of the cell cycle. Each phase of the cycle is regulated by specific cyclin-dependent kinases (CDKs), their regulatory partners, the cyclins, and CDK inhibitors. Image from [177].

Mitotic entry is determined by the activation of CDK1, after its association with its mitotic partners, cyclin A and B. CDK1 activates other mitotic kinases, such as PLK1, Aurora A and B, and NIMA-related kinases (NEK). Altogether, these kinases drive the phosphorylation of thousands of proteins with the subsequent changes affecting every cellular compartment, followed by chromosome separation and finally, cell division [178,179]. This process takes place in five differential phases known as prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, CDK1 activity rises in the cytoplasm, leading to centrosome separation [180]. In parallel, activated CDK1-Cyclin B complex is translocated to the nucleus inducing chromosome condensation and nuclear membrane permeabilization. In prometaphase, the nuclear envelope is broken-down, and the condensed chromosomes get in touch with the microtubules (MT) [181]. During prometaphase, the E3 ubiquitin ligase APC/C^{CDC20} induces the degradation of cyclin A, but CKD1 remains active by its interaction with cyclin B [182]. After that, each chromatid attaches bilaterally at the kinetochores with the MT emerging from the centrosomes. This results in the alignment of chromosomes at the centre of the cell forming the mitotic spindle, a symmetric structure characteristic of metaphase [183]. After chromosome alignment, mitotic exit initiates. APC/C^{CDC20} ubiquitylates B-type cyclins, promoting their proteasome degradation. Also, protein phosphatases start reverting the activity of CDK1 by extensive dephosphorylation. In the transition from metaphase to anaphase, APC/C^{CDC20} promotes the activation of separase protein, leading to sister chromatids segregation toward the opposite poles of the cell [184]. This process is strictly controlled by MT dynamics and require the forces emitted by motor proteins. During telophase, once the chromosomes are separated, two new functional nuclei are formed [185]. At the end, the remaining cyclin B is eliminated, CDK inactivated, and the process succumbs with the formation of two identical daughter cells at G1 phase [179].

Despite the extensive regulatory machinery, mistakes during the process can emerge due to external factors or merely at random. To ensure that only healthy cells with intact genomes proliferate, cell cycle checkpoints have evolved as a response upon the detection of these errors. The G1/S checkpoint is activated when damage is detected during interphase. Cells with damaged DNA are restricted to enter S phase, preventing the propagation of genetic errors [186]. The intra-S-phase checkpoint reduces DNA replication velocity during S-phase to minimize replication errors and to ensure complete duplication [187]. The spindle assembly checkpoint (SAC) is the major cell-cycle control mechanism. It ensures that chromosomes are equally segregated by the detection of mistakes in the spindle formation that may result in abnormal chromosome partitioning and aneuploidy [188]. When one of these checkpoints is activated, cell cycle is arrested until the damage is resolved. If the harm is unbearable, other processes such as apoptosis, senescence or

mitotic catastrophe are activated to prevent propagation of mistakes. Failure of these mechanisms may lead to the accumulation of alterations that can result in uncontrolled proliferation and genomic instability [174].

1.3.2. Mitotic inhibitors in clinical use and clinical development

Cell cycle and mitotic alterations that result in uncontrolled cell proliferation are well characterised as a hallmark of human cancer [189]. For this reason, mitosis and mitotic regulators have been considered valuable targets for anti-cancer therapy development for many years. Microtubule poisons, agents that affect MT dynamics and assembly, have shown clinical benefits in the treatment of solid tumors for over 25 years, particularly taxanes. Nonetheless, severe related side effects, such as neurotoxicity and myelosuppression, and the emergence of resistances, limit their clinical use [190]. Still, the idea of inhibiting mitosis and MT dynamics remains compelling, and efforts are now being focused on the development of therapies that target distinctive features of tumor cells to reduce undesirable side effects. **Figure 12** shows some key mitotic components that are usually overexpressed in tumor cells, for which targeting agents have been developed. Among the most common targets used to inhibit mitosis are spindle microtubules, mitotic kinases, motor proteins, and multi-protein complexes, such as the involved in SAC signalling [174].

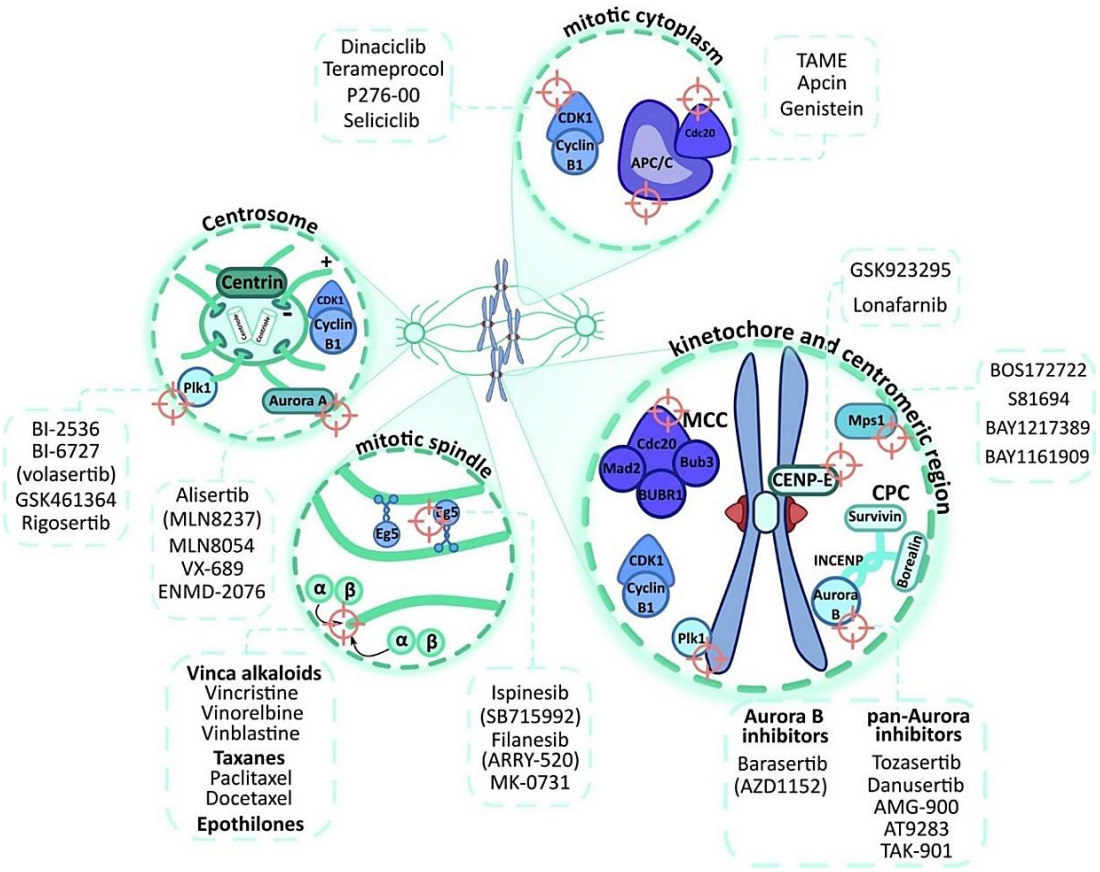

Figure 12. Summary of key mitotic proteins and their current promising targeted therapies. The image shows multiple molecular components involved in several steps of mitosis, including spindle microtubules, mitotic CDK, other non-CDKs kinases, motor proteins and multi-protein complexes, such as the implicated in the SAC, which are targets of numerous anti-tumoral agents that are already approved or are under evaluation. Image from [191].

It is important to highlight that in the specific case of PCa, it was described some years ago, that during progression towards CRPC, a transcriptomic reprogramming characterised by an overexpression of M-phase cell cycle genes was taken place [192]. Furthermore, the same authors observed that this transcriptomic switch was directly orchestrated by the androgen receptor. This suggests that mitotic proteins may display important roles in the progression of CRPC and may become effective therapeutic targets for the treatment of patients at this lethal stage of the disease.

Targeting microtubules

Microtubule targeting agents (MTA) were the first drugs discovered for targeting mitosis. They are products of natural origin and the best characterised is paclitaxel, which was isolated in 1971 as a result of a project from the American National Cancer Institute, looking for new anticancer drugs [193]. MTA affect MT dynamics and assembly by blocking MT polymerization (i.e., vinca alkaloids, such as vincristine, vincristine or vinorelvine) or

depolymerisation (i.e., taxanes, such as docetaxel, paclitaxel or cabazitaxel) [194] resulting in activation of the SAC due to unattached kinetochores, leading to mitotic arrest and eventually cell death [195]. Although these agents have shown important clinical benefits, they are related with severe side effects. MTAs are not specific for tumor cells as they can affect proliferating healthy tissues, such as the bone marrow, causing myelosuppression, or neurotoxicity, as MT are essential for neuronal transport [196].

In the case of PCa, taxanes are the most used chemotherapeutic agents. Docetaxel was approved for the treatment of mCRPC in 2004, changing radically the therapeutic scope of these patients, who until that moment were treated with palliative therapies [109]. This agent binds to β-tubulin, stabilizing MT by polymerization [197]. Cabazitaxel is a second-generation taxane, used as second-line therapy for those patients that developed docetaxel resistance. It is also a MT stabilizing agent but is more potent than docetaxel and performs lower affinity for P-gp efflux pumps, which are related with multidrug resistance [110]. Furthermore, quite recently, the improved performance displayed by taxanes in PCa patients compared with other chemotherapeutics has been linked to their ability to target some other pathways important for PCa progression [197].

Targeting Cyclin-dependent kinases

When CDK1 binds to its regulatory partner cyclin B, the complex starts to phosphorylate its targets allowing the entry and progression of mitosis. Although alternations in CDK1 have not been commonly described in cancer, the kinase was shown to be essential for tumor initiation and progression [198]. Furthermore, overexpression of B-type cyclins and consequently, elevated CDK1 activity, was shown to increase the susceptibility to some cancer types [199]. CDK1 inhibition has demonstrated interesting results in some cancer preclinical models as lymphoma, liver, and breast cancer [200,201]. Furthermore, CDK1 hyperactivation in PCa has been linked with increased AR phosphorylation and stabilization, which may contribute to CRPC development [202,203]. However, the poor response rates observed in several clinical trials along with the severe adverse events related with the essential role of CDK1 in normal cell division questioned the utility of CDK1 inhibitors as an anticancer therapy. Recent evidence suggests that tumor cells are more dependent on interphase CDKs to proliferate, such as CDK2, 4 and 6, than normal cells, and are deregulated in a wide variety of tumors [204]. Abemaciclib, ribociclib, and palbociclib are the only FDA approved CDK inhibitors. They act against CDK4/6 and were authorized for the treatment of ER positive, HER2 negative breast cancer patients [205]. Results of the performance of these agents from PCa clinical trials are still awaited [206].

Targeting Aurora kinases

Aurora kinases (AURKs) belong to the Aurora family of Serine/Threonine kinases, which is formed by three members: Aurora A, B and C. They have similar substrate specificities but different subcellular localizations, which allows them to perform distinct mitotic tasks [191]. Aurora Kinase A (AURKA) localizes at the centrosomes and is essential for mitotic entry, centrosome maturation, duplication, and mitotic spindle assembly. Aurora Kinase B (AURKB) is found as a component of the chromosome passenger complex (CPC), which participates with SAC functions regulating proper MT-kinetochore attachment and appropriate chromosome segregation [207]. The role of Aurora Kinase C (AURKC) is not fully elucidated. It seems to share some function with AURKB based on its association with the CPC, and has been also related with meiotic division [207,208]. Abnormal AURK functions are associated with cell division defects and aneuploidy. Both, AURKA and AURKB have been described to be amplified and overexpressed, respectively, in many tumor types, including PCa [174]. Moreover, overexpression of AURKA has been associated with PCa progression to CRPC [209,210]. Dozens of AURKA inhibitors have been developed during the last years, but clinical development of most of them has been stopped due to lack of efficacy and/or toxicity. Alisertib is considered one of the most potent AURKA inhibitors and is by far the most clinically advanced. It has been tested in over 40 clinical trials and has showed interesting results in breast, lung, ovarian and PCa [211–213]. However, alisertib seems to display anticancer effects only on a subset of tumors, so the identification of biomarkers of response to AURK inhibitors could accelerate their clinical development.

Targeting Polo-like kinase 1 (PLK1)

Polo-like kinases are serine/threonine kinases that form a family of five proteins. PLK1 is the member that has been studied in more detail. It is activated by AURKA and plays critical roles during mitosis, participating in centrosome maturation, bipolar spindle assembly, chromosome segregation, regulates the APC complex and in the initiation of cytokinesis [191]. Non-mitotic roles, such as apoptosis inhibition and regulation of cancer invasiveness have also been attributed to PLK1. Overexpression of this protein have been observed in several solid tumors, including PCa and haematological malignancies, and correlates with poor prognosis [174]. Furthermore, its expression is elevated in androgen independent PCa cells, likewise AURKA, suggesting that it might play a role in castration resistance [214]. Giving these, PLK1 is considered a potential target for cancer therapy and multiple inhibitors have been developed showing promising preclinical results [174]. However, none have demonstrated significant clinical activity and caused severe adverse events. Moreover, it is important to highlight that other family members, such as PLK2 and PLK3 have tumor

suppressor functions thus, PLK1 inhibitors must be highly specific to avoid undesirable effects [215]. BI2536 was one of the first PLK1 inhibitors that were developed. It showed promising results in phase I trials, but the outcomes from the subsequent phase II trials were quite disappointing [216]. BI2767 (volasertib) is the improved derivative of BI2536 and have showed some encouraging clinical results, especially in acute myeloid leukaemia patients, having reached phase III clinical development [217]. This increase sensitivity of leukaemia cells to PLK1 inhibition compared to solid tumors may be due to their higher proliferation rates [218].

Targeting the SAC

The SAC is a signalling cascade activated by cells to monitor correct attachment of each kinetochore to MTs in order to prevent chromosome miss-segregation. If an error is detected, chromosome separation is delayed, and cells are arrested in mitosis until chromosomes are properly orientated in the spindle [219]. It is well known that many cancer types have disrupted, although not completely absent, SAC functioning, which causes chromosome instability (CIN), aneuploidy, and drives malignancy. Further disruption of SAC signalling would generate extreme levels of CIN exceeding the adaptation capacity of cancer cells, generating a therapeutic opportunity [174]. Monopolar spindle protein 1 (MPS1) is a key component of the SAC cascade and along with MAD1, BUB3 and BURB1, is recruited to the kinetochore in the presence of improperly attached MT [220]. The important role of this protein in the initiation of SAC signalling, along with its observed overexpression in several tumors and its relation with poor prognosis [215,221,222], encouraged the development of agents against MPS1 activity. To date, several inhibitors have been identified and are under preclinical and clinical evaluation [223,224]. Moreover, based on the mechanism of action of SAC inhibitors, it is thought that their combination with MTAs could enhance the generation of chromosome segregation errors above the tolerated threshold of cancer cells [225]. In fact, the combination of paclitaxel with MPS1 inhibitors (BAY1217389 and BAY1161909) is being evaluated in phase I clinical trials. Initial results showed considerable toxicities and it was discussed that improved dose determination for two agents with overlapping toxicities is needed [226].

1.3.3 Understanding mitotic cell death

Cancer cell exposure to antimitotic drugs leads to mitotic arrest due to SAC activation that is usually followed by apoptotic cell death through a caspase-dependent mechanism [227]. However, some cells can undergo a process called “mitotic slippage”, in which cells are able to exit mitosis without undergoing cell division, returning to G1 phase in an aneuploidy state [228]. Several studies of cell lines at single cell level have shown that different cell types, and even genetically identical cells, can follow different mitotic cell fates, suggesting that non-genetic factors are influencing this process [229,230]. To explain this behaviour, the “competing-networks” model (**Figure 13**) was proposed. This model suggests that mitotic cell fate is determined by two competing and independent pathways, one involving the activation of cell death signalling and the other cyclin B degradation [227]. Both networks have thresholds and cell fate would depend on which one is reached first. If the death threshold is breached first, cell would die in mitosis. On the other hand, if cyclin B is degraded rapidly and CDK1 activity falls below the mitotic exit threshold first, mitotic slippage occurs [231]. It is important to mention that cell fate after slippage is not totally elucidated. It is suggested that after mitotic exit, cells can either die in interphase, undergo G1 arrest or start a new cell cycle as tetraploid [229]. In the last case, the subsequent mitosis would be tetrapolar, which should end in cell death. However, some cancer cells have the ability to align multiple centrosomes into a bipolar spindle, generating viable daughters and this, in the context of therapy resistance, might be noteworthy [232].

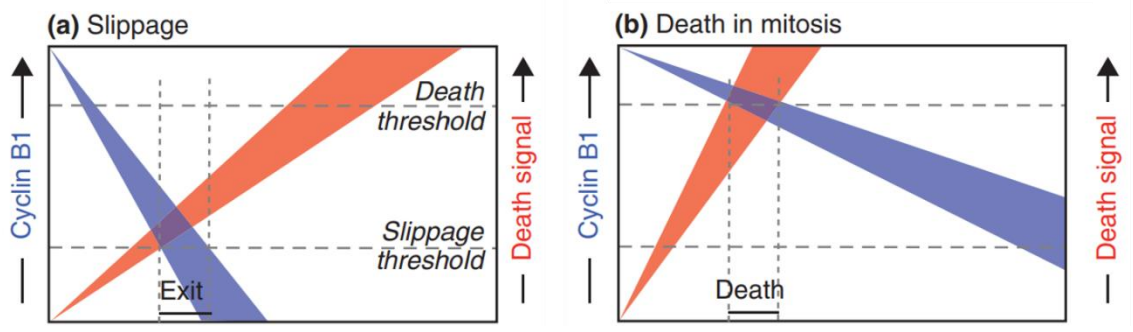

Figure 13. Schematic representation of the competing networks model. During prolonged mitotic arrest, cyclin B levels (blue) slowly reduce. When cyclin B levels fall below the threshold needed to maintain CDK1 activity, cells leave mitosis without dividing. In parallel, death signals accumulate (red). In this case, if death threshold is reached before mitotic slippage, cells suffer mitotic death. Graph **(A)** represents slippage-prone cells due to fast fall of cyclin B levels. Graph **(B)** represents death-prone cells, as death signals accumulate faster than cyclin B degradation. Image adapted from [233].

Mitotic cell death generally occurs through mitochondrial apoptosis, which is controlled by the BCL-2 protein family [234]. This family is composed by a set of anti-apoptotic members including BCL-2, BCL-xL and MCL-1, which antagonize apoptosis by blocking the activity of the BH3 pro-apoptotic proteins BIM, BID, BAD, BAX and NOXA [235]. These BH3 members work mediating mitochondrial membrane permeability, allowing the activation of caspases, proteases that mediate cellular death [236]. Some of these anti-apoptotic proteins are thought to be, at least in part, responsible of the mitotic slippage that some cells undergo after the treatment with antimitotic agents. For this reason, the combination of antimitotics with inhibitors of the BCL-2 family members was proposed as good strategy to enhance antimitotic-induced cell death [237]. Navitoclax/ABT737 is a potent inhibitor of several BCL-2 antimitotic family proteins, including BCL-2, BCL-xL and BCL-W [238] and has already shown to potentiate taxanes cytotoxic effects in several preclinical models [239–241]. Nonetheless, to generate rational combinations, it is important to take into account that the BCL-2 family members seem to have redundant functions. MCL-1 is an important member of the BCL-2 family, which levels during interphase are high, while in mitosis are naturally reduced due to an imbalance between its synthesis and proteolysis. That would explain why cancer cells did not respond to navitoclax treatment alone, whereas during mitotic arrest, induced by an antimitotic drug, navitoclax was able to effectively accelerate apoptotic activity through inhibition of BCL-xL added to the current low levels of MCL-1. The same authors also described that in cells with low levels of BCL-xL, MCL-1 degradation during the mitotic arrest was enough to cause cell death, while in cells with higher levels of BCL-xL, the degradation of MLC-1 was not sufficient [242]. However, the whole picture of mitotic death regulation is highly complex, with multiple components implicated. Further research focused on mitotic cell fate are needed to understand the underlying networks and to generate new opportunities to turn the balance in favour of tumoral cell death.

1.4. Kinesin superfamily (KIF)

Kinesins are a superfamily of motor proteins that use the energy of ATP hydrolysis to travel along microtubule tracks and carry out their functions related with cell division and intracellular transport of cellular components [243]. To date, there are 45 described genes codifying for kinesins in mammals that are organized into 14 subfamilies based on their catalytic core motor domain homology [244]. Among the 45 kinesins, 16 have been shown to have important functions during mitosis (**Figure 14**), participating in spindle assembly, chromosome alignment and segregation, and cytokinesis [243].

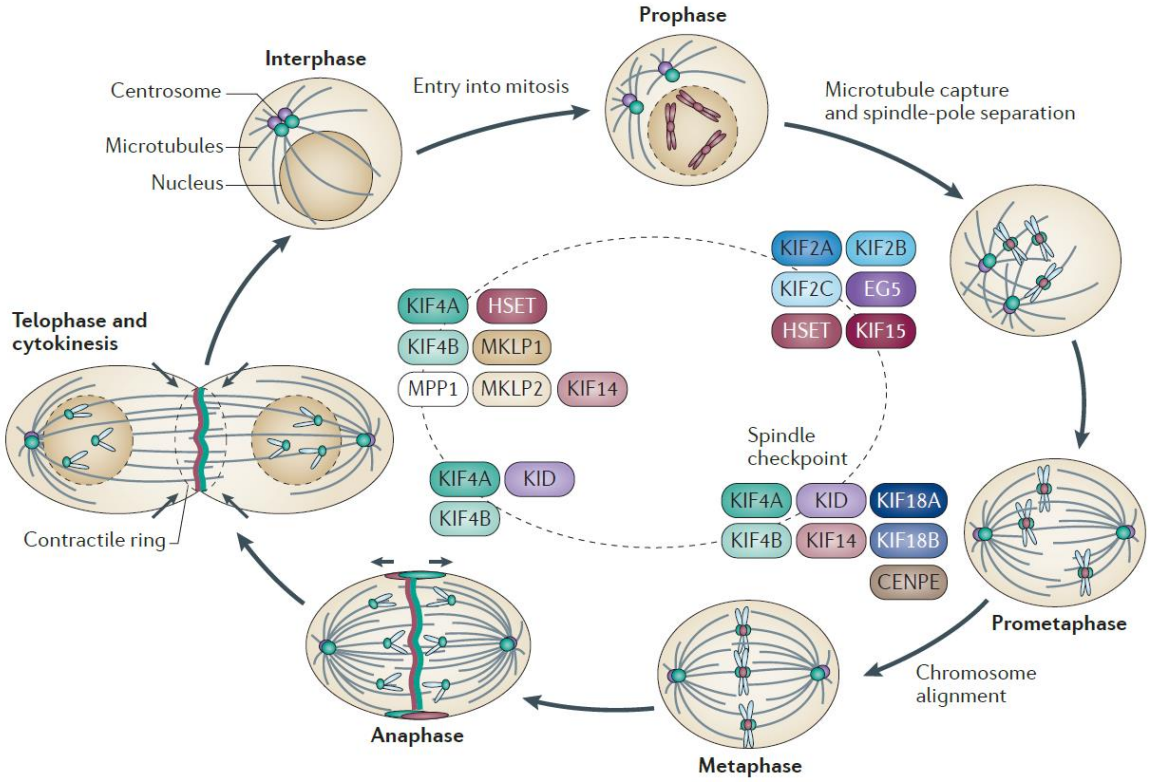

Figure 14. Main mitotic kinesins at each stage of mitosis and cytokinesis. Kinesins are positioned at the stages at which they display their functions. Of note, some kinesins have a single function, while others are involved at different mitotic phases. Also, some of these motor proteins have non-mitotic functions, such as neuronal transport. Figure from [243].

1.4.1. Kinesins and cancer

During the last decades, increasing evidence suggests that kinesins play critical roles in the development of human cancers and many of them have been implicated with malignancy and drug resistance of several tumors. Thus, targeting kinesins seems a promising anticancer strategy and several inhibitors of KIF proteins have enter clinical studies, alone or in combination with other drugs [245].

Kinesin family member 11 (KIF11, also known as Kinesin-5, Eg5, or kinesin spindle protein, KSP) and centromere-associated protein E (CENPE) are the two kinesins with the most advanced inhibitors that have reached clinical trials. **KIF11** will be analysed in detailed in section 1.4.2. **CENPE** is an essential protein during progression from metaphase to anaphase, orchestrating chromosome congression at the metaphase plate [246]. Furthermore, it is also important for proper chromosome alignment, as it interacts with other proteins that mediate SAC signalling [247]. GSK923295, a CENPE allosteric inhibitor, showed antitumor activity in several preclinical models [248] and results from a phase I clinical trial were encouraging [249]. Furthermore, on the bases that CENPE inhibition results in CIN [250], it was demonstrated that CENPE inhibitors could be especially effective in tumors with impaired SAC signalling [251].

HSET (also known as KIFC1) has been shown to be essential during bipolar spindle formation and dynamics and in cytokinesis. Overexpression of this protein has been related with the development of brain metastasis in lung cancer patients and with poor prognosis in ovarian cancer [252,253]. Inhibition of HSET demonstrated to be particularly effective in cells containing multiple centrosomes. These cells, in order to avoid multipolar mitosis and cell death, need to cluster their chromosomes into two poles, and HSET is a key protein in this process. These findings make this kinesin particularly attractive for anticancer therapy, as it is essential for cancer cells, but not for normal cells [232,254]. However, not many companies have focused their attention on this protein yet and there are not many inhibitors available[243].

KIF15, also called HKLP2, belongs to the kinesin-12 family. It plays critical roles in the spindle formation by crosslinking parallel and antiparallel microtubules [255] and it has also been implicated with intracellular transport of cellular components [256]. Its overexpression has been related with progression and poor prognosis of several cancers such as lung, pancreatic, breast and hepatocellular carcinoma [257–260]. Moreover, it has been related with the development of hormonal therapy resistance in prostate and breast cancer and with the development of CRPC [256,261,262]. Furthermore, during the last years more attention is being focused on the study of this kinesin, as several works suggest that it might be, at least in

part, responsible of the development of resistance to KIF11 inhibitors, maybe due to some overlapping functions [263,264]. Some KIF15 inhibitors have been developed and have shown promising results in preclinical works, especially in combination with KIF11 inhibitors [255]

Kinesin-13 family members have microtubule depolymerase activity that contributes to the regulation of microtubule dynamics during mitotic progression [265]. The most studied members of this family are KIF2A, KIF2B and mitotic centromere-associated kinesin (MCAK or KIF2C). The activity of these kinesins is regulated by mitotic kinases such as PLK1, AURKA and AURKB, highlighting the coordination between kinesins and general cell cycle progression [266–269]. **KIF2A** is crucial for the preservation of spindle bipolarity and it also seems to play important roles in neuronal development, as KIF2A(-/-) mice displayed severe neuronal phenotypes [270]. Moreover, overexpression of this kinesin has been correlated with poor prognosis and more aggressive disease in several types of cancer [271–274]. **KIF2B and MCAK** regulate the interaction between kinetochores and MTs, correcting improper MT attachment thus, preserving genome stability. MCAK is the best characterise member of the family and its deregulation and overexpression has been linked with increased malignancy, metastasis [275] and chemotherapy resistance due to an increase rate of MT detachment from centrosomes [276]. In the particular case of PCa, MCAK overexpression has been related with CRPC development [277]. Altogether, MCAK is considered an interesting cancer drug target and some inhibitors have been developed [278]. However, more studies to improve their specificity and proof-of-principle assays in animal models are needed before their clinical development. Moreover, a possible function redundancy between MCAK and KIF2A/B that could overcome MCAK inhibition should be investigated [243].

KIF4A and KIF4B (or chromokinesins) are two very similar but distinct kinesins that play essential roles regulating spindle dynamics during anaphase and in cytokinesis [279]. Alterations in either of both kinesins result in binucleated cells and other mitotic defects [280]. The most recent studies have been focusing on KIF4A. Its overexpression has been related with enhanced proliferation, metastasis development and poor prognosis in colon, esophageal, breast and hepatocellular carcinoma [281–284]. Moreover, it has been also associated with endocrine therapy resistance and poor BCR-free survival in PCa patients [285,286]. Although they seem to be implicated in several oncological processes and could be considered promising therapeutic targets, their redundant functions make that both proteins would have to be targeted simultaneously. Further investigation is required however, other proteins with narrower mitotic functions such as CENPE or KIF11 are currently more attractive targets [243].

In prometaphase, during a process known as chromosome congression, the chromosomes move from the spindle poles towards the metaphase plate. KIF18A, KIF18B and KID (KIF22) are three kinesins with important functions during this process [243,287]. **KIFI18A** and **KIF18B** belong to kinsin-8 family. KIF18A plays a role in the correction of chromosome positioning influencing the dynamics of the plus-end of kinetochore microtubules. Depletion of this protein leads to longer spindles, and the activation of the mitotic checkpoint due to misaligned chromosomes [287]. Its overexpression has been linked with increase proliferation, invasion and metastasis in several types of cancer [288–290] and also with poor response to endocrine therapy in ER positive breast cancer [291]. Based on that, KIF18 is considered to have anticancer therapeutic potential and some inhibitors has been developed [292]. Moreover, very recently, it has been shown that KIF18A is essential for CIN tumor cells but not in normal diploid cells and this could be exploited to specifically target tumors with these characteristics [293]. The mitotic function of KIF18B has been more recently discover and little is known about its implication in cancer [294]. However, its overexpression has already been linked with cell proliferation and tumor progression in several tumors [295–297]. **KID** is a monomeric motor protein that generates forces to push chromosomes towards the spindle equator [298]. Depletion of this kinesin leads to a delay in metaphase-to-anaphase transition but eventually the cells are able to divide normally [299]. This points out to a possible redundant function, which excludes KID as a potential target [243].

Cytokinesis is the process in which, once chromosomes have been segregated, the two daughter cells separate. More than two hundred proteins take part in that process, of which the members of kinesin-6 family, MKLP1 (KIF23), MKLP2 (KIF20A), MPP1 (KIF20B) and KIF14 (member of kinesin 3-family) have major roles [300]. **Kinesin-6 family** members have a unique structural insertion in the motor domain which is thought to be valuable for the development of highly specific inhibitors [243]. Depletion of these kinesins leads to defects or failure of cytokinesis, with generation of binucleated or multinucleated cells and subsequent apoptosis [301,302]. Overexpression of the three of them has been related with tumor progression and poor prognosis in several types of cancer and are considered potential targets for drug development [303–308]. Importantly, they do not seem to share specific functions, which is an advantage for the development of anticancer agents, as the inhibition of one would not be replaced by the others [280]. Similarly, **KIF14** depletion also leads to endoreduplication, multinucleated cells and eventually, cytokinesis failure and apoptosis [309]. Its overexpression has also been related with tumor progression and metastasis and is considered to have prognostic value in several cancers [310,311].

There is a large amount of literature describing the implication of kinesins in cancer and many of them are considered potential therapeutic targets, for which inhibitors has been

developed. However, the evaluation of some of these proteins as specific drug target is very challenging. Many of them have multiple functions during cell cycle and careful validation is needed in order to avoid undesirable side effects. For this reason, CENPE and KIF11, which have more limited and better-defined roles during mitosis, have the most clinically advanced anticancer agents of all the kinesin superfamily members[243].

1.4.2. A special focus on Kinesin family member 11

1.4.2.1. KIF11 structure and function

KIF11 (also known as Kinesin-5, Eg5, or kinesin spindle protein, KSP) is encoded by Kif11 gene of approximately 62 Kb, located at 10q24.1 chromosomal region. It belongs to Kinesin-5 family, and like all kinesins, it is formed by a head, a stalk, and a tail domain (**Figure 15**) [312]. As other members of this family, KIF11 monomers self-assemble into homotetrameric structures composed by two dimers arranged in an antiparallel fashion. Each dimer is formed by an helical coiled coil, followed by the head domain [313]. This is a unique characteristic of kinesin-5 family members, and it is thought to be essential to carry out their function during mitosis [314]. The head domain, also known as the motor or catalytic domain, is located at the N-terminus of the protein. It contains the catalytic region, responsible of the hydrolysis of ATP to generate mechanical energy [315], and a conserved microtubule binding site that keeps the protein attached to these structures [316]. At the C-terminal end of the head domain is located the neck linker, a region conserved between kinesins that is believed to interact directly with the catalytic domain, influencing the processivity and directionality of the homotetramer [317]. At the C-terminal end of the protein is found the tail domain, which as the head domains, contains microtubule binding sites that help with KIF11 movement along microtubules by ensuring correct MT-protein interaction [318]. Finally, at the central part of the protein is located the stalk region, that contains the structural elements needed for the tetramerization of the protein. Within this region is found the bipolar-assembly (BASS) subdomain, essential for the formation of the bipolar homotetrameric complex. The deletion of this region results in monomeric KIF11 proteins [319]. Moreover, BASS subdomain is thought to be needed for the transference of the forces generated by the catalytic domain through all the monomers, required for sliding apart the antiparallel MT [320].

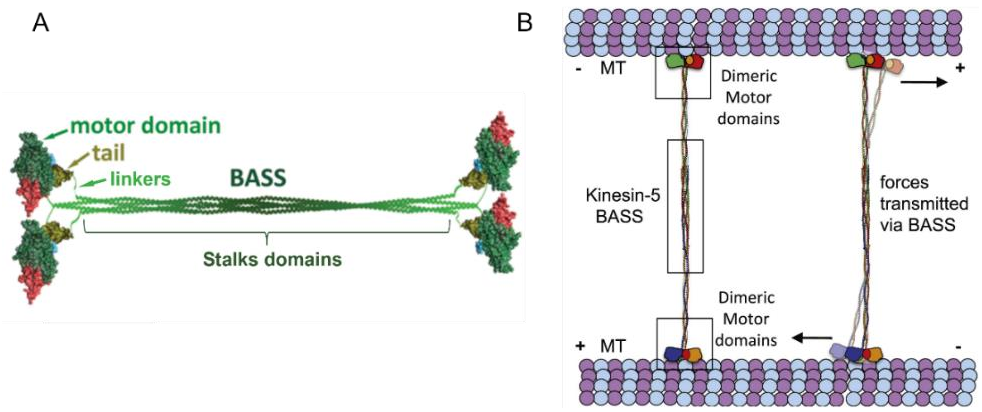

Figure 15. Schematic description of KIF11 domains and its arrangement in crosslinked microtubules. **(A)** Representation of full-length tetrameric KIF11 showing the motor and tail domains at the end of the bipolar structure connected by the central stalk, which consists of four helixes with the BASS domain at the central part. **(B)** Model of KIF11 filaments interacting with two antiparallel MTs, crosslinking, and sliding them apart. Arrows indicate the direction of KIF11 movement. Images adapted from [319,320].

The main KIF11 function is to participate in the formation of the bipolar spindle during early prophase and to assist chromosome segregation in late mitosis (**Figure 16**). Its ability to form homotetramers allows the establishment of a bridge between antiparallel microtubules that are moved in opposite directions by the action of the motor heads. During prophase, KIF11 generates the necessary forces to slide apart the microtubules that extend from each duplicated centrosome, driving spindle bipolarity [321]. Later in mitosis, once the centrosomes are at opposite poles and the chromosomes are aligned at the metaphase plate, KIF11 continues sliding apart the duplicated centrosomes, cooperating with bipolar spindle maintenance, elongation and eventually, chromosome segregation [322].

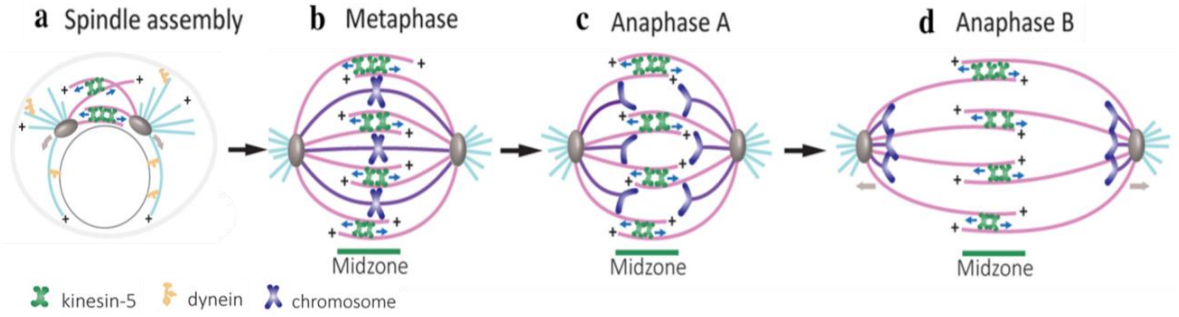

Figure 16. Schematic representation of KIF11 roles during mitosis. (A) KIF11 provides the necessary forces to slide apart antiparallel spindle MT in prophase. The direction of the movement of KIF11 and the spindle poles are indicated by the arrows. **(B)** At metaphase, the bipolar spindle is formed, and chromosomes are congressed at the metaphase plate. KIF11 crosslinks antiparallel MT at the midzone and stabilize the spindle. **(C, D)** During anaphase, sister chromatids lose cohesion and are pulled to opposite spindle poles by the elongation of the spindle, leading to chromosome segregation. This elongation is generated by the forces produced by motor proteins attached to the cortex, such as dynein, and the forces exerted by KIF11 on antiparallel MT at the midzone. Image adapted from [319].

KIF11 depletion or pharmacological inhibition generates the formation of the characteristic monopolar spindles (**Figure 17**), as the centrosomes are not able to separate and migrate to the opposite poles [323]. This induces a mitotic arrest due to SAC activation that usually leads to cell death by apoptosis [324].

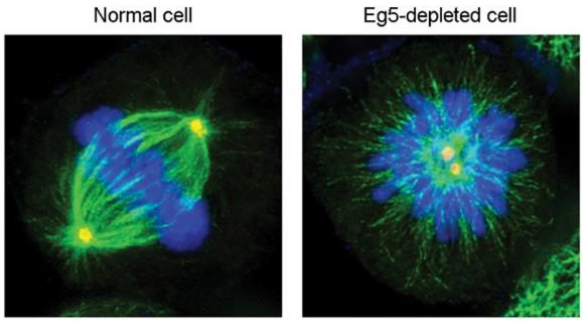

Figure 17. Formation of monopolar spindles after KIF11 depletion. Immunofluorescence microscopy images of MT (green), γ-tubulin (red), and chromosomes (blue) in normal metaphase (left) and KIF11 depleted HeLa cells (right). Image adapted from [324].

During the last years, KIF11 has also been implicated with other functions, such as the regulation of microtubule-end dynamics, by its recently described MT polymerase activity [325,326]. It has also been reported to contribute in the transport of secretory proteins from the Golgi apparatus to the cell membrane [327] and to have a role during polypeptide synthesis, participating in ribosome transport along MT [328].

1.4.2.2. KIF11 mitotic regulation

As previously mentioned, mitosis progression is accurately coordinated by phosphorylation/dephosphorylation events carry out by specific mitotic kinases and phosphatases. In this context, KIF11 is not an exception, and its amino acid sequence contains multiple phosphorylation sites that regulate its activation and localization throughout the cell cycle [323]. It has been reported that eukaryotic kinesin-5 members share a conserved sequence located in the C-terminal tail, known as “BimC box”, which contain several phosphorylation sites for CDK1 [329]. KIF11 phosphorylation by CDK1 at T926 during early mitosis allows localization of the motor protein at centrosomal and spindle MT [330]. Moreover, CDK1 mediated phosphorylation of KIF11 is also required for its interaction with other proteins, such as dynein, through the dynactin subunit $p150_{glued}$ [331]. PLK1 also plays a role in KIF11 regulation during mitosis, in this case indirectly, through the phosphorylation of NECK9 kinase. Activated NECK9 targets NECK6/NECK7, which then phosphorylate KIF11 at Ser1033, once it is localized at centrosomes, allowing its motor function and the establishment of the bipolar spindle [332,333]. The proposed model supports that CDK1, at prophase, phosphorylates KIF11 promoting its localization and accumulation at

centrosomal MT and then, phosphorylation by Neck6/7 kinases promotes motor activity, centrosome separation, and eventually spindle bipolarity [334].

Apart from the above-mentioned KIF11 tail domain phosphorylation that triggers its spindle localization, it has been reported that phosphorylations at the catalytic domain, near the ATP-binding pocket, regulate its motor activity. In this case, Src kinase would be the responsible of KIF11 post-translational modification, specifically at Y211, Y125 and Y231 sites. It is believed that these phosphorylations do not affect the ability of the protein to localize or bind with MT, but rather they tune the motor activity to achieve optimal spindle bipolarity [335]. It has been shown that AURKs also phosphorylate KIF11 stalk region at S543 site in *Xenopus laevis*, although its role in mitosis is not totally clear. This AURK phosphorylation site is also found in mammalian KIF11 however, a role in mitosis has not been reported [336].

Although the factors that promotes KIF11 localization and activity on the mitotic spindle are quite well defined, little is known about KIF11 fate at the end of mitosis. A couple of quite recent works have studied this matter. Liu *et al*. demonstrated that PP2A/B55α phosphatase complex plays an important role in mitotic exit by KIF11 dephosphorylation. The same authors also demonstrated that PP2A depletion results in abnormal KIF11 function and delayed mitotic exit [337]. Remarkably, He and colleagues showed that PTEN phosphatase also plays an important role in KIF11 function during mitosis balancing its phosphorylation levels. In fact they showed that PTEN depletion caused KIF11 hyperphosphorylation, affecting its association with MT and centrosomes, leading to mitotic abnormalities [338].

Another protein that has been shown to be essential for KIF11 localization at the spindle is TPX2, which is a spindle assembly factor that binds to MT and recruits other proteins important for this process [339]. TPX2-KIF11 interaction was reported to be essential for KIF11 localization at spindle MT and for the correct activity of the motor protein. Consistent with this, perturbation of this interaction triggers KIF11 abnormal activity, with the production of imbalanced forces that lead to cells with highly disorganized multipolar spindles [340,341].

1.4.2.3. KIF11 and cancer

Since the discovery of the important role that KIF11 plays in cell proliferation and given the significance of this process in cancer development, efforts have been focused on the study of the implication of this protein in tumorigenesis. In fact, overexpression of KIF11 in transgenic mice was demonstrated to lead to extensive aneuploidy and genetic instability, both hallmarks detected in the majority of tumors, which ultimately resulted in a high incidence of neoplastic transformation [342]. In line with this, KIF11 has been found

overexpressed in multiple malignant tumors and this high expression was correlated with several clinico-pathological characteristic. **Table 6** shows a summary of several tumor types in which KIF11 expression has been found to be altered.

Table 6. KIF11 expression and its correlation in multiple malignancies

Tumor type	KIF11 status	Refs.
Breast cancer	KIF11 overexpression was correlated with poor prognosis and high stage of the disease	343
Neuroblastoma	KIF11 overexpression was associated with poor outcome	344
Prostate cancer	KIF11 expression levels correlated with higher PSA, metastatic disease, and docetaxel resistance	345,346
Lung adenocarcinoma	High KIF11 expression correlated with worse PFS and OS	347
Oral cancer	KIF11 expression was found in cancer tissue but not in healthy oral epithelia and its levels were associated with poor prognosis	348
Bladder cancer	KIF11 expression was associated with higher tumor grade, stage, and poor prognosis	349,350
Gallbladder cancer	KIF11 was found overexpressed in tumor tissue compared with paired normal tissue	351
Renal carcinoma	KIF11 expression was significantly higher in tumor tissues compared with non-cancerous adjacent tissues, and was associated with tumor grade and worse OS	352
Pancreatic cancer	KIF11 was overexpress in tumor compared to normal tissue and associated with higher histological grade	353
Hepatocellular carcinoma	High KIF11 expression was associated with worse OS and DFS	354,355
Ovarian cancer	High expression of KIF11 was significantly correlated with shorter OS and PFS	356
Glioblastoma	KIF11 was upregulated in glioma tumors, negatively correlated with OS, and associated with chemoresistance	357
Chronic myeloid leukaemia	KIF11 was described to be highly expressed in blast crisis chronic myeloid leukaemia	358

Abbreviations: PSA, prostate specific antigen; PFS, progression free survival; OS, overall survival; DFS, disease free survival

All these data indicate that KIF11 might be playing a crucial role in tumor development and aggressiveness. It is considered a typical oncoprotein with promising prognostic value and an interesting target for the development of anticancer drugs [359].

1.4.2.4. KIF11 inhibitors

All the cumulative aforementioned data suggest that KIF11 is an essential protein for cell proliferation and mitosis, specifically during the establishment and maintenance of the bipolar spindle. Moreover, multiple evidence points out its important role in carcinogenesis and tumor progression. All these, along with the fact that KIF11 has been reported to be selectively overexpressed in tumors compared with healthy tissues, and the rapidly increasing emergence of resistance to classic MTAs, makes of KIF11 an attractive mitotic

target that is currently under investigation. The first small molecule inhibitor against KIF11 that was discovered was monastrol, in 1999 [360]. This served as a proof of concept for the development of a new type of antimitotic inhibitors that do not target microtubules and therefore, the severe adverse events related to them could be avoided. As mentioned, KIF11 is minimally expressed in adult tissues, including neurons, and therefore, the classic neurotoxicity associated to MTA is expected to be minimal. Since then, numerous KIF11 inhibitors with improved specificity and efficacy have been developed and several of them have reached clinical development as anticancer drugs (**Table 7**) [361].

Table 7. KIF11 inhibitors in clinical development. Table adapted from [361]

Inhibitor	Company	Phase	Condition	Refs.
Arry-520	Array Biopharma	I/II	Soli; MM, AML, MDS	NCT02384083, NCT01372540, NCT02092922, NCT01989325 NCT00637052, NCT00462358, NCT00821249, NCT01248923
LY2523355	Eli Lilly	I/II	Solid, leukemia, ovarian, NSLCLC, PC, CRC, gastric, esophageal, HNSCC, BC, SCLC	NCT01358019, NCT01416389 NCT01214655, NCT01059643 NCT01214629, NCT01214642, NCT01025284
4SC-205	4SC	I	Solid, lymphoma,	NCT01065025
ALN-VSP02	Alnylam	I	HCC	NCT01158079, NCT00882180
Ispinesib	GSK	I/II	Burkett lymphoma; RCC, HNN, PC, ovarian, BC, NSCLC, CRC, HCC, melanoma	NCT00354250, NCT00089973 NCT00119171, NCT00095628 NCT00607841, NCT00363272 NCT00097409 NCT00098826 NCT00136578, NCT00101244 NCT00096499, NCT00085813 NCT00095953, NCT00095992 NCT00103311, NCT00169520
AZD4877	AstraZeneca	I/II	Solid, lymphoma, NHL, bladder, transitional, urethra, RCC	NCT00471367, NCT00661609 NCT00613652, NCT00486265 NCT00389389, NCT00460460
SB 743921	Cytokinetics	I/II	Solid, NHL, HL	NCT00343564, NCT00136513
ARQ 621	ArQule	I	Solid	NCT00825487
MK-0731	Merck	I	Solid	NCT00104364

Abbreviations: MM, multiple myeloma; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; NSLCLC, non-small-cell lung carcinoma; PC, prostate cancer; HNSCC, head and neck squamous cell carcinoma; BC, breast cancer; SCLC, small cell lung cancer; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; NHL, non-Hodgkin lymphoma; HL, Hodgkin lymphoma

All agents listed **Table 7** in demonstrated high ability to inhibit the enzymatic activity of the motor protein *in vitro* and were highly efficient impairing tumor growth in several *in vivo* preclinical models. In general, results from phase I trials were encouraging, as the agents were well tolerated, being among the most common side effects neutropenia, anemia and in some cases, liver toxicity. Remarkably, unlike MTAs, none of them showed any sign of neurotoxicity. However, in subsequent clinical development, only few of them showed promising results [361]. Among the most encouraging agents is Filanesib (Arry-205), which demonstrated high efficacy in heavily pre-treated multiple myeloma patients, especially when administered in combination with other compounds [362]. Also, 4SC-205 showed remarkable signs of efficacy when administered at a continuous dosing of 20 mg/day in patients with several solid malignancies and lymphomas [363].

The underlying reasons responsible for the apparent failure of some of these agents remain unknown. However, the results suggest that KIF11 inhibitors could be more effective in certain types, or even subtypes of tumors. Moreover, given the essential function of the protein during mitosis, a continuous dosing scheme could improve their efficacy. By doing so, the specific agent would be longer in the serum of the patient, increasing its chance to hit the target in a greater proportion of cells at a given moment, especially critical in slow dividing tumors [361]. Finally, it is important to consider that the development of therapeutic strategies based on rational combinations could also increase the efficacy of these mitotic inhibitors.

2. HYPOTHESIS AND OBJECTIVES

Prostate cancer is the second most common type of cancer and the fifth cause of cancer-related deaths among men, with almost 1.4 million of new cases and more than 375000 deaths worldwide in 2020. Although important advances have led to improved early detection and survival, 20 to 30% of patients that are diagnosed with localized disease will experience recurrence. Given the important role that androgens play in the progression of PCa, for several decades androgen deprivation therapy has been the “gold standard” treatment for advanced PCa. Despite its initial beneficial effects, almost all patients progress to CRPC through complex and heterogeneous mechanisms usually implying the AR. Novel AR antagonists, such enzalutamide, have shown significant survival benefit for CRPC patients. However, despite initial response, most patients eventually develop resistance. Therefore, understanding the molecular bases underlying the development of CRPC and these resistances is an urgent need in order to identify therapeutic targets to treat the disease at this lethal stage.

Multiple findings indicate that aberrations in cell cycle and mitosis are hallmarks of the development of multiple malignancies. Proteins involved in these processes are considered promising therapeutic targets for the development of new anticancer drugs. In fact, some of these agents, such as taxanes, are being used in the clinical practice. However, although they perform some survival benefits, they display severe dose-limiting toxicities, and resistances unavoidably appear. In the specific case of PCa, during the last years increasing evidence has shown that a transcriptomic reprogramming orchestrated by the androgen receptor and characterized by the upregulation of M-phase genes is taking place during its progression toward CRPC. In previous work from our group, it was validated for the first time that this transcriptomic reprograming was reproduced at protein level using high-throughput quantitative proteomics. From this analysis, KIF11 outstood as a promising candidate that might play a key role during this process.

Hypothesis: Taking into account the above-mentioned evidence our hypothesis is that mitotic proteins and, more specifically KIF11, might play a crucial role in PCa progression to CRPC and therefore, its inhibition could be an effective therapeutic strategy to treat this lethal disease. To validate this hypothesis, we set the following objectives:

Objective 1. To evaluate the implication of KIF11 in the progression of PCa towards castration resistance and its potential value as a prognostic biomarker.

Objective 2. To study the therapeutic efficacy of KIF11 by 4SC-205 for the treatment of CRPC using several *in vitro*, *ex vivo* and *in vivo* preclinical models.

Objective 3. To unveil novel potential molecular mechanisms by which KIF11 might be participating in CRPC progression.

Objective 4. To decipher and evaluate new therapeutic strategies for the treatment of CRPC patients based on rational combinations of KIF11 inhibition with the standard of care of PCa patients.

3. MATERIAL AND METHODS

3.1. PCa gene expression datasets and bioinformatics tools

A detailed *in silico* analysis was carried to study KIF11 expression at different stages of PCa progression using several datasets: GSE35988 (Benign n=18; PCa n=54; mCRPC n=33), GSE3325 (Benign n=6; PCa n=7; mCRPC m=6); GSE32269 (Benign n=11; PCa n=47; mCRPC n=26), GSE21034 (Benign n=29; PCa n=130; mCSPC n=14; mCRPC n=12) were used to analyse KIF11 expression in the indicated groups. Transcriptional information was obtained using GEO2R tool. Expression data of PCa patients with different Gleason scores and biochemical recurrence were obtained from TCGA database collection. To study the correlation between AR and KIF11 gene expression, data were obtained from GSE70769, GSE29079 and TCGA using R2 Genomics website. For disease and progression-free survival analysis, data were retrieved from TCGA and GSE21034 datasets using cBioPortal website. Datasets GSE104935 and GSE51873 were used to analyse KIF11 expression in enzalutamide-resistant PCa models. All bioinformatics tools and databases are specified in **Table 8**.

Table 8. Human databases and bioinformatics tools used in this study

Name	Description	N.	Geo/Ref.	Tool
TCGA	Surgical resection biospecimens from patients diagnosed with prostate adenocarcinoma	493	TCGA data portal	R2Genomics cBioPortal
Taylor *et al.*	Tumoral, metastatic and matched normal samples obtained from patients treated by RP. Clinical and pathologic data were maintained prospective.	179	GSE21034	Geo2R cBioPortal
Grasso *et al.*	28 benign prostate tissues, 59 localized PCa obtained by RP and 35 CRPC obtained by rapid autopsy	122	GSE35988	Geo2R
Varambally *et al.*	Frozen tissue blocks from benign prostate tissue, clinically localized PCa and hormone refractory metastatic tissue	19	GSE3325	Geo2R
Balk *et al.*	22 hormone treatment naïve prostate tissues isolated from biopsies and 29 bone metastases from CRPC obtained from bone marrow biopsies	51	GSE32269	Geo2R
Dunning *et al.*	Primary PCa from RP with matched benign tissue, CRPC from chTURP, independent benign samples from HoLEP	94	GSE70769	R2Genomics
Li *et al.*	Sensitive and resistant C4-2 cells selected by continuous treatment with enzalutamide 1 µM	4	GSE104935	Geo2R
Arora *et al.*	LNCaP xenograft and its derivative with acquired resistance to 2nd generation antiandrogen enzalutamide	15	GSE51873	Geo2R

Abbreviations: N., number of samples; RP, radical prostatectomy; CRPC, castration resistant prostate cancer; chTURP, channel transurethral resection of the prostate; HoLEP, holmium laser enucleation of the prostate

3.2. Human PCa samples

Formalin-fixed paraffin-embedded (FFPE) tissue samples obtained after radical prostatectomy were collected from the biobank of the Pathology Unit of Vall d'Hebron University Hospital (Barcelona, Spain). Tumoral area was obtained by laser microdissection in order to optimise the number of cancer cells for later mRNA analysis. Additionally, adjacent healthy tissues from PCa patients were used as control for expression analysis. Ten years follow-up of the selected patients since the date of the surgery allowed the stratification of these patients depending on their disease progression, based on the development or not of metastatic disease (**Table 9**).

FFPE tissues from patients with benign prostatic conditions and PCa naïve tumors isolated by TURP or RP respectively were obtained from the biobank of the Pathology Unit of Vall d'Hebron University Hospital (Barcelona, Spain). FFPE samples from CRPC patients were kindly provided by the Pathological Anatomy of the Marche Polytechnic University (UNIVPM, Ancona, Italy). Additionally, FFPE biopsy tissues were obtained from treatment naïve patients with advanced disease. After ADT initiation, patients were followed up during treatment and their samples were divided into two groups named as responders (those who still respond after two years of treatment) and resistant (those who developed CRPC before one year of ADT treatment). All patients gave their written inform consent and samples were obtained by a specialised urologist under the approval of the hospital's ethical committee. Detailed information about patient's clinical characteristics is specified in **Table 10.**

Table 9. Clinico-pathological characteristics of patients used for mRNA expression analysis

	PCa M0	PCa M1
No. of samples	14	5
Age (years)*	63.3 (55-70)	65.6 (61-69)
PSA (ηg/ml)*	11.4 (5.4-20.5)	12.2 (3.1-23.5)
GS 6	3	-
GS 7	7	3
GS 8	2	-
GS 9	2	2
GS 10	-	-

Abbreviations: PSA, prostatic specific antigen; GS, Gleason score; M0, no metastatic disease after 10 years follow-up; M1, metastatic disease

Table 10. Clinico-pathological characteristics of patients included in the immunohistochemical analysis

	BPH	PCa-naïve	CRPC**
No. of samples	8	8	8
Age (years)*	-	63.1 (55-68)	79.6 (75-86)
PSA (ηg/ml)*	-	12.3 (7-20.2)	18.5 (10-35)
GS 6	-	4	-
GS 7	-	4	-
GS 8	-	-	-
GS 9	-	-	5
GS 10	-	-	2

Abbreviations: PSA, prostatic specific antigen; BPH, benign prostatic hyperplasia; CRPC, Castration resistant prostate cancer; GS, Gleason score; * Values are represented as mean (range); ** Gleason score was not available from one CRPC patient

	Responders	Resistants
No. of samples	9	9
Age (yr)*	67.1 (58-75)	73.5 (52-85)
Disease Free Months*	42.8 (24.7-78.2)	9.1 (6.8-12.8)
GS 6	-	-
GS 7	1	-
GS 8	5	2
GS 9	1	7
GS 10	2	-

Abbreviations: yr, years; PSA, prostatic specific antigen; GS, Gleason score; *Values are represented as mean (range);

3.3. Cell culture

3.3.1. Commercial cell lines

The non-tumorigenic prostate epithelium derived cell line RWPE-1 and human PCa cell lines LNCaP, PC-3, Du145 and 22Rv1 were obtained from American Type Culture Collection (ATCC). LNCaP AI cell line, is an androgen independent (AI) derivative from the above-mentioned LNCaP. It was generated by being continuously maintained in androgen-depleted conditions and was a kindly gift from Dr. Anna C. Ferrari (Icahn School of Medicine at Mount Sinai, NY, USA). Murine PCa cell line NPp53 was a kindly gift from Dr. Aytes (IDIBELL, Barcelona, Spain). Cell line characteristics are summarised in **Table 11**. RWPE-1 cells were cultured in Keratinocyte Serum Free Medium (K-SFM) with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF) (Thermo Fisher Scientific; #17005075). LNCaP AI cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated charcoal (Sigma-Aldrich; C6241) stripped serum (CSS) and 1% insulin-transferrin-selenium supplement (Life Technologies). LNCaP, PC3, Du145, 22Rv1 and NPp53 cells were cultured in RPMI-1640 (Biowest; L0500) with 10% heat-inactivated fetal bovine serum (FBS). All media were supplemented with 2 mM L-glutamine (X0550), 1% penicillin-streptomycin solution (L0022), 1% MEM non-essential amino acids (X0557) and 1% sodium pyruvate (L0642) (all from Biowest). All cultures were maintained at 37°C in a humidified saturated atmosphere of 5% CO_2. All cells were expanded and stored in liquid nitrogen and were periodically tested for mycoplasma contamination.

Table 11. Characteristics of prostate cancer cell lines used in this thesis. Table adapted from[364]

Name	Origin	Doubling time	AR RNA	AR protein
Normal				
RWPE-1	HPE cells from the peripheral zone	120 h	Yes	Yes
Hormone naïve				
LNCaP	Lymph node metastasis	28-60 h	Yes	Yes
Castration resistant				
LNCaP AI	LNCaP derivative, obtained after prolonged androgen withdrawal	~ 55 h	Yes	Yes
NPp53	Derived from Nkx3.1 $Cre^{ERT2/+}$; $Pten^{flox/flox}$; $Tp53^{flox/flox}$ transgenic mouse PCa tumors	~ 30	Yes	Yes
Du145	Brain metastasis	~ 33 h	No	No
PC3	Vertebral metastasis	~ 34 h	No	No
22Rv1*	CWR22R castrated xenograft line	35-40 h	Yes	Yes
C4-2	LNCaP castrated xenograft line	48 h	Yes	Yes

Abbreviations: HPE, human prostate epithelial; AI, androgen independent
*AR-V7 expressing cell line

3.3.2. Patient derived cells

Primary cultures were derived from tumor cells obtained from core needle biopsies of patients from the Urology Unit at Vall d'Heborn Hospital following Dr. Paciucci's protocol. Briefly, cells were maintained in DMEM-12 medium (Biowest; L0093) supplemented with 7% heat-inactivated FBS, 1% penicillin-streptomycin solution, 1% MEM non-essential amino acids, 2 mM L-glutamine, 0.6% glucose (Sigma-Aldrich; G8270). Additionally, 1 mg/mL transferrin (Sigma-Aldrich; T5158), 1 µg/mL putrescine (Sigma-Aldrich; #51799), 0.3 µM sodium selenite (Sigma-Aldrich; S5261), 100 µM hydrocortisone (Sigma-Aldrich; H0888), 0.25 mg/mL insulin (Life Technologies; #12585014), 20 ηg/uL EGF (Thermo Fisher; PGG6045), 10 ηg/mL FGF (Thermo Fisher; PHG0266), 200 ηg/mL vitamin E and 200 ηg/mL vitamin A (Sigma-Aldrich) were freshly added every time. Cultured flasks were previously coated with collagen (Corning, #354249) and were not manipulated during the first week, as it is the time the cells take to adhere. After that, medium was changed, cells were amplified and stored in liquid nitrogen. Primary cultures were maintained at 37 ºC in a humidified saturated atmosphere of 5% CO_2. Written informed consent was obtained from all patients and all procedures were approved by the ethical committee of the Hospital (CEIC number PR(AG)96/2015).

3.4. Gene expression analysis

3.4.1 RNA extractions

RNA from tumor tissue: total RNA was obtained using miRNeasy Mini Kit (Quiagen; #217504) following manufacturer's instructions. Briefly, 2-3 mm^3 tumor pieces were cut into smaller fragments on dried ice and were homogenized using FastPrep 24 Matrix tubes (MP Biomedicals; #116913050) with 700 µl of QIAzol lysis reagent (Quiagen; #79306) in the Fast-Prep®-24 instrument during 30 seconds (sec) at 6.5 r/p for 3 times. The resulting homogenates were centrifuge for 5 minutes (min) at 1200 rpm at 4 ºC, to eliminate tissue debris. Supernatants were transferred to 1.5 ml tubes and 140 µl chloroform were added to proceed with the standard protocol of the kit. **RNA from FFPE tissue** was collected using miRNeasy FFPE kit (Qiagen; #217004), following manufacturer's instructions. Before RNA extraction from this samples, tumoral areas were laser microdisected. **RNA from cell lines:** cell line total RNA was extracted using miRNeasy Mini Kit (Quiagen; #217504). Briefly, cells were trypsinized and spun down for 5 min at 1200 rpm. Cell pellets were washed once with 1x phosphate-buffered saline (PBS) and 700 µl of Quiazol lysis reagent were added to each sample. Then, total RNA was extracted following manufacturer's instructions.

In all cases, total RNA samples were eluted using 20-30 µl of RNase-free water and were subjected to DNasa treatment. RNA concentration and quality was measured by Nanodrop 2000 and samples with an A260:A280 ratio between 1.8 and 2 were accepted for further analysis.

3.4.2. RNA retrotranscription

1 µg of total RNA was subjected to reverse transcription using 1 µl of RevertAid H Minus Reverse Transcriptase (200/U) and 1 ul of random hexamer primers (Thermo Fisher; K1632). In order to avoid RNA template secondary structures, these reagents were incubated at 65 ºC for 5 min. The reaction was carried out in a thermal cycler using the following conditions: 5 min at 25 ºC followed by 60 min at 42 ºC and finished with 5 min at 70 ºC to stop the reaction. The resulting cDNA was stored at -20 ºC until analysis.

3.4.3. Real-time quantitative PCR

RT-qPCR was performed using Power SybrGreen Master Mix (Applied Biosystems; A25742) and reaction was carried out in ABI Prism 7900HT equipment. Primer sequences (**Table 12**) to detect specific gene expression were used at a final concentration of 0.1 µM.

TATA-box binding protein (TBP) was utilized as the endogenous control for normalization and $2^{(-\Delta\Delta Ct)}$ method was applied for relative quantification of gene expression [365].

Table 12. Primer sequence of genes analysed by RT-qPCR

Gene name		Primer sequence (5' to 3')	Amplicon size
KIF11	Fw:	AAAACAACAAAGAAGAGACAATTCC	93 nt
	Rv:	CAGATGGCTCTTGACTTAGAGGT	
AR	Fw:	CCATCTTGTCGTCTTCGGAAATGTTATGAA	143 nt
	Rv:	AGCTTCTGGGTTGTCTCCTCAGTGG	
AR-V7	Fw:	CCATCTTGTCGTCTTCGGAAATGTTATGAA	125 nt
	Rv:	TTTGAATGAGGCAAGTCAGCCTTTCT	
PSA	Fw:	TCCGTGACGTGGATTGGT	83 nt
	Rv:	CAGGGTTGGGAATGCTTCT	
TMPSRSS2	Fw:	GGTAAACTCTCCCTGCCACA	78 nt
	Rv:	TACTCCAGGAAGTGGGGATG	
TBP	Fw:	GAACATCATGGATCAGAACAACA	87 nt
	Rv:	ATAGGGATTCCGGGAGTCAT	

Abbreviations: nt, nucleotides

3.5. Protein analysis

3.5.1 Protein extraction and quantification

From cell lines: Total lysates were obtained using Pierce RIPA buffer (Thermo Fisher Scientific; #8900) supplemented with phosphatase cocktail inhibitors (Sigma-Aldrich: P5726, P0044) and 1X EDTA-free complete protease inhibitor (Roche; #11836170001). Samples were kept on ice for 1 hour with 20 sec vortex every 15 min. For protein extraction from the nuclear and cytoplasmatic cellular subfractions, NE-PER™ Nuclear and Cytoplasmic extraction Kit (Thermo Scientific; #78833) was used, following manufacturer instructions. Samples were stored at -20 ºC until immunoblot analysis. *From xenograft tumors:* Tissues were cut into small pieces with a lancet and immersed in RIPA buffer 1x with phosphatase cocktail inhibitors (Sigma-Aldrich: P5726, P0044) and 1X EDTA-free complete protease inhibitor (Roche; #11836170001). Tumors were desegregated with a tissue homogenizer (Bead Ruptor 12 (Omni, Inc.)) for 10 sec three times and sonication at an amplitude of 100A for 5 sec. Finally, protein extracts were obtained after 15 min centrifugation at 16000 g at 4 ºC. Protein concentration was determined with BioRad DC protein assay (Bio-Rad Laboratories) following manufacturer's instructions. Samples were stored at -20 ºC until analysis.

3.5.2. Immunoblot

Protein extracts from whole cell lysates (40-100 µg of protein) and from nuclear/cytoplasmatic fragmentation (15 µg of nuclear proteins and 25 µg of the cytoplasmic fraction) were resolved in NuPAGE 8-12% Bis-Tris gels at 120V and transferred onto PVDF membranes, blocked for 1h with 5% bovine serum albumin (Sigma-Aldrich; A7906) or 5% non-fat milk (PanReac AppliChem; A0830) and then probed at 4 ºC overnight with the specified antibodies (**Table 13**). Membranes were washed three times and incubated for 1h with the corresponding secondary antibodies (1:5000; Sigma-Aldrich) at room temperature. Final protein detection was obtained with ECL Western Blot System (GE Healthcare; #28980926).

3.5.3 Immunoprecipitation (IP)

C4-2 and 22Rv1 cells were collected and lysed using an IP lysis buffer formulated with 50 mM Tris HCl, 300 mM NaCL, EDTA 2 mM, 10 % glycerol, 1% triton 0.1 % SDS and EDTA-free protease inhibitor (Roche; #11836170001). The lysates were kept on ice for half an hour, vortexing briefly every 5 min and were centrifuged at maximum speed during 20 min at 4 ºC. Supernatants were collected. A preclearing step of the lysates with Rabbit IgG and sepharose bead powder (#P3391, Sigma-Aldrich) resuspended in ethanol 70% was performed to reduce background. Samples were centrifuge for 10 min at 14000g and 4 ºC. Cleared lysates were collected, quantified as explain above, and a small amount was taken to use as input (25 µg). For each IP, 500 µg of protein were separated in different 1.5 ml tubes and incubated at 4 ºC overnight with the suitable antibody at the appropriate concentration (specified in **Table 13**), or with the same amount of Rabbit IgG. Then, the immune complexes were incubated with 25 µl of sepharose beads for 2h at 4 ºC and pulled down with a full speed spin. The supernatants were carefully discarded and the sepharose beads were washed three times with IP lysis buffer. Finally, 1x loading buffer and 30 µl of IP lysis buffer were added to the beads and boiled for 5 min. Beads were pulled down, discarded and the proteins present in the supernatants were analysed by western blot as explained previously (section 3.5.2). All the antibodies and concentrations used in the IPs are specified in **Table 13**.

3.5.4 Immunohistochemistry

Tumor tissues isolated from mice were fixed with 10% formalin for 24h, washed and stored in 1x PBS until they were paraffin embedded at the Vall d'Hebron Hospital's Pathology Unit. FFPE human samples were obtained as explain in section 3.2. Tissue samples were deparaffinised at 60 ºC overnight and re-hydrated through alcohol washes. Antigen retrieval was performed using pH 6-9 citrate buffer, during 4 min at 115 ºC using a pressurized heating chamber. Endogenous peroxidase blockage was carried out and then samples were incubated with primary antibodies (**Table 13**) overnight at 4 ºC. Specimens were then incubated during 30 min at room temperature with anti-Rabbit secondary antibody (Dako; K4003). Colour reaction was achieved using diaminobenzidine (Dako; K3468) and counterstained with haematoxylin (PanReac AppliChem; #253949.1611). KIF11 staining in human samples was reviewed and scored by an uropathologist following the H-score system. Briefly, each sample was scored based on the percentage of cells with a specific staining intensity. Intensity values range from 0 to 3, corresponding to no staining, low, moderate, or high staining, respectively. The staining intensity score for each sample was calculated using the following formula: H-score = [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] [366].

Percentage of cells with positive staining for phosphorylated-Histone H3 (pH3) was evaluated considering the number of tumor cells in ten different fields per sample and counting the number of pH3 positive cells using ImageJ Software. Hematoxylin and eosin (H/E) staining was performed following the conventional protocol at the pathology unit of the hospital.

Table 13. List of antibodies used in this thesis

Antibody	Reference	Manufacturer	Dilution
Primary antibodies for Western Blot			
KIF11	[329]	-	1:3000
KIF11	sc-365593	Santa Cruz	1:500
AR	#5153	Cell Signaling	1:1500
Caspase-3	#9665	Cell Signaling	1:3000
Cleaved Caspase-3	#9661	Cell Signaling	1:1000
PARP	#9542	Cell Signaling	1:3000
Cleaved PARP	#5625	Cell Signaling	1:1000
Cyclin B1	#05-373	Merk Millipore	1:1000
p-Histone H3 (Ser10)	#9701	Cell Signaling	1:1000
CREB-1	sc-186	Santa Cruz	1:200
β-Actin	sc-47778	Santa Cruz	1:10000
α-Tubulin	T9026	Sigma Aldrich	1:5000
Primary antibodies for immunoprecipitation			
AR	sc-7305	Santa Cruz	2.5 µg Ab/ 500 µg protein
AR	06-680	Merk Millipore	2.5 µg Ab/ 500 µg protein
KIF11	23333-1-AP	Proteintech	2 µg Ab/ 500 µg protein
Secondary antibodies for Western Blot			
Anti-Rabbit IgG	A0545	Sigma Aldrich	1:5000
Anti-Mouse IgG	A9044	Sigma Aldrich	1:5000
Primary antibodies for immunohistochemistry			
KIF11	HPA006916	Sigma Aldrich	1:2000
p-Histone H3 (Ser10)	#9701	Cell Signalling	1:200
AR	ab133273	Abcam	1:150
Secondary antibodies for immunohistochemistry			
Anti-Rabbit HRP	K4003	Dako	K4003

Abbreviations: Ab, antibody

3.6. Transient transfection

PCa cells were transfected with synthetic small interfering RNAs (siRNAs) (**Table 14**) at a final concentration of 25 ηM for KIF11 siRNA and 50 ηM for AR siRNA. Transfection was performed using Lipofectamine® 2000 (Invitrogen; #11668-019). To obtain transfection vesicles, lipofectamine and siRNAs were diluted and mixed in OPTIMEM (Gibco; #31985070) serum-free medium and incubated for 20 min. Then, cells were plated with the corresponding culture medium and transfected at the same time (reverse transfection). After overnight incubation, medium was changed to avoid lipofectamine toxicity. The efficiency of siRNA silencing was verified by western blot and RT-qPCR.

Table 14. List of siRNAs used in this thesis

Gene	siRNA	Sequence (5'-3')	Reference	Supplier
LaminA	siRNA LaminA	GGACCUGGAGGUCUGCUGU	[367]	Dharmacon
	siRNA LaminA_as	ACAGCAGACCUCCAGGUCC		
KIF11	siR-KIF11	CUAGAUGGCUUUCUCAGUA	[368]	Sigma-Aldrich
	siR-KIF11_as	UACUGAGAAAGCCAUCUAG		
AR	siR-AR	-	sc-29204	SCBT
	siR-AR_as	-		

Abbreviations: SCBT, Santa Cruz Biotechnology

3.7. Generation of stable cell line

3.7.1. Constructs for KIF11 overexpression

For KIF11 overexpression system, KIF11 pDONR221 was obtained from Harvard PlasmID Database and cloned into pINDUCER20 (Addgene; plasmid #44012) using Gateway LR Clonase II (Invitrogen; #11791-020) following the manufacturer's protocol. pINDUDER-GFP was a kindly gift from Dr. Arango's group (IRB, Lleida, Spain) and was used as negative control in functional assays. The obtained vectors were sequenced using pINDUCER20 forward primer (5'-ACCTCCATAGAAGACCC-3') in the High Technology Unit at Vall d'Hebron Research Institute, to ensure that they contained the correct sequence.

3.7.2. Lentiviral production and transduction

Lentiviral particles were produced following an adapted protocol from Naldini *et al*. [369]. Briefly, to obtain viral particles $4x10^6$ HEK-293T cells were seeded in a 100-mm plates the day before transfection. After 24h, cells were co-transfected with the plasmid of interest (pINDUCER20-KIF11 or pINDUDER-GFP), the envelope pMD2.G (Addgene; #12259) and packaging psPAX2 (Addgene; #12260) lentiviral vectors using Lipofectamine® 2000 (Invitrogen; #11668-019). Medium was change 6h post-transfection to avoid lipofectamine toxicity. After 48h, viral supernatants were collected and filtered through a 0.45 µm syringe filter. LNCaP cells were transduced with the fresh viruses and 5 µg/ml of polybrene (Millipore) were added to enhance transduction efficiency. After 24h of incubation, medium was changed, and cells transduced with the plasmid of interest were selected with 500 µg/ml G418 (Gibco; #10131027) for 72h. KIF11 overexpression was induced with 0.1 µg/ml doxycycline (Thermo Scientific; #15510554) and confirmed by RT-qPCR and western blot before used in experiments. Plasmid employed in this work are specified in **Table 15**.

Table 15. Plasmids and vectors used in this thesis

Vector	Description	Manufacturer
pDONR221 KIF11	KIF11 coding sequence cloned	Harvard Plasmid Database
pINDUCER20	Tet-inducible lentiviral vector for ORF expression	Addgene plasmid #44012
pINDUCER_GFP	Tet-inducible lentiviral vector for GFP expression	-
pINDUCER_KIF11	Tet-inducible lentiviral vector for KIF11 expression	-
pMD2.G	2nd generation lentiviral envelope particle	Addgene plasmid #12259
psPAX2	2nd generation packaging plasmid	Addgene plasmid #12260

3.8. Cell assays

3.8.1. Cell proliferation assay (crystal violet)

For drug sensitivity assays, PCa cell lines (LNCaP, LNCaP AI, PC3, Du145, 22Rv1 and C4-2) and the non-tumoral epithelial prostate cell line RWPE-1, were seeded at a density of 2×10^3–5×10^3 cells per well on 96-well plates (n=3/condition). The day after, cells were treated with 4SC-205 in a concentration range between 0 and 100 ŋM. After 72h of drug exposure, cells were fixed with formaldehyde 4% solution (VWR International BVBA; #9713) during 20 min at room temperature and stained with 0.5% crystal violet (PanReac AppliChem; A0691). After washing with H_2O, crystals were dissolved with 15% acetic acid (Fisher Scientific; #10041250) and optical density was measured by spectrophotometry at 590 ŋm.

3.8.2. Cell proliferation assay (cell counting)

To determine the effect of KIF11 overexpression on PCa cell growth under androgen depleted conditions, cell proliferation was analysed by cell counting. LNCaP pIND_GFP and pIND_KIF11 cells were seeded into six-well plates at a density of $8x10^4$ cells/well in CSS-RPMI-1640 medium. To induce vector expression 0.1 µg/mL doxyxycline (Thermo Scientific; #15510554) were added to each well. Cells were counted with Trypan blue (Nano Entek; #734-2676) and reseeded once a week during 4 weeks.

3.8.3. Colony formation assay

Colony formation capacity under KIF11 inhibition was analysed in PCa cell lines (LNCaP, LNCaP AI, PC3, Du145, 22Rv1) and the non-tumoral normal epithelial prostate cell line RWPE-1. Cells were seeded onto six-well plates at low density ($5x10^2$-$1x10^3$; n=3/condition). After 24h, cells were treated with 4SC-205. Medium was refreshed every 3 days. After 10 days, cells were fixed with 4% formaldehyde solution, stained with 0.5% crystal violet, photographed and scored. Differences in colony formation capacity were assessed by comparing the number of colonies formed by each cell line under 4SC-205 treatment with their vehicle-treated control.

3.8.4. Mitosis and cell death assays

LNCaP and Du145 cell lines were seeded onto 24-well plates at a density of $2.5x10^4$ and $1.5x10^4$ respectively. After 24h, cells were treated with either vehicle dimethyl sulfoxide (DMSO) (PanReac AppliChem; A3672) or 4SC-205 at 6 ŋM in the case of LNCaP and 25 ŋM for Du145. 12, 24 and 48h post-treatment, cells were simultaneously stained with Hoechst 33258 dye (1 µg/ml) (Sigma-Aldrich; #94403) and propidium iodide (2.5 µM) (PI; Thermo Fisher Scientific; P1304MP) and photographed using a fluorescence microscope (Nikon Eclipse TE2000-S). Mitosis and cell death quantification was made from 5 representative images of each well (n=2 replicates/condition). Mitotic index was calculated using Hoechst staining, as number of cells with condensed chromosomes/total cells [370]. For the apoptotic index, cells with positive PI staining were considered apoptotic, as this dye cannot enter the plasma membrane of living cells [371].

3.8.5. Cell cycle analysis

Du145 cells were seeded at a density of $1x10^6$ in 100-mm dishes and synchronized in G1/S phase using 2 mM of the nucleoside thymidine for 24h. Then, cells were washed three times with PSB and medium was refreshed. Approximately 8h after thymidine removal, cells were treated either with vehicle or 25 ŋM 4SC-205. Flow cytometry analysis was performed after 12 and 24h of drug exposure. For that aim, cells were trypsinized, counted and $1x10^6$ were fixed with 30% 1x PBS and 70% ice cold ethanol solution. The mixture was slowly added while shaking the cells to avoid aggregates formation. Afterwards, cells were stained with a freshly prepared solution of 38 mM sodium citrate (Sigma-Aldrich; W302600), 500 µg/ml propidium iodide (Thermo Fisher Scientific; P1304MP) and 10 mg/ml of RNasa A (Sigma; R-5503) and incubated overnight at 4 ºC. Samples were then analysed by flow cytometry using FACScalibur machine (BD Biosciences) and data were processed with FCS Express

by De Novo software (BD Biosciences). Exact same procedure was followed to analyse the reversibility of the effects of the inhibitor, with the only difference that cells were treated with the inhibitor for 12h, then washed and refreshed with new medium for 12 additional hours before harvested and processed for cell cycle analysis.

3.8.5. Drug combination studies

To determine drug toxicity upon the combination of 4SC-205 and enzalutamide (Quimigen; HY-70002), 22Rv1 (5 x 10^3 cells/well), C4-2 cells (4 x 10^3 cells/well) and NPp53 cells (1 x 10^3 cells/well) were seeded into 96-well plates (n=3 replicates/condition). The day after, cells were treated with concentrations from 1 to 20 ηM of 4SC-205 and from 1 to 30 µM of enzalutamide, using five different concentrations for each drug to identify synergy. 72h later, cells were fixed with 4% formaldehyde and stained with 0.5 crystal violet. Cell proliferation inhibition upon the combination-treated cells was compared with vehicle and single drug-treated ones. Full dose response curves were obtained for each drug and the combination index (CI) for all pairwise combinations was determined following the Chou-Talalay method [372] with Compusyn Software (ComboSyn Inc.).

3.8.6. Spheroid viability assay

Primary cultures were derived from tumor cells isolated from core needle biopsies as explained in section 3.2.2. Cells derived from three patients with advanced and aggressive PCa (patient characteristics are specified on **Table 16**) were used to generate tumor spheroids as previously described [367]. Briefly, cells were seeded at a density of 2x10^5 cells/well in non-adherent six-well plates (coated with 0.5% agarose non-supplemented DMEM-F12 medium) in serum-free DMEM-F12 supplemented with 20 ng/ul EGF (Thermo Fisher; PGG6045), 10 ng/ul FGF (Thermo Fisher; PHG0266) and 0.4% B27 vitamin (Gibco; #17504044). After 24h, spheroids were treated with 4SC-205 at the indicated concentrations. After 72h treatment, spheroids were collected, pelleted at 100 g for 5 min, washed once with PBS and disaggregated with 0.5 ml of 1X StemPro® Accutase® Cell Dissociation Reagent (Thermo Fisher Scientific; #15323609). For spheroid viability determination, MTS assay was performed on disaggregated cells. PMS and MTS reagents (Promega; G1112) were mixed at a ratio of 1:20 and 10 µl of the mixture were added to each well (96 well-plates) containing 100 µl of disaggregated cells (n=3 replicates/condition). Several MTS absorbance measures at 490 ηm were taken during 2-5h of incubation using an Epoch Microplate Spectrophotometer (Biotek).

Table 16. Patient-derived primary cultures used in this thesis

Patient ID	Serum PSA (ηg/ml)	Gleason	CRPC	Metastasis
VHP-1	1106	9 (5+4)	No	M1
VHP-2	39	10 (5+5)	Yes	M0
VHP-3	12	9 (4+5)	Yes	M0

Abbreviations: VHP, Vall d'Hebron Hospital patient

3.9. Protein stability and degradation analysis

To determine AR protein stability under KIF11 depletion, $3.5x10^5$ 22Rv1 cells and $2.5x10^5$ C4-2 cells were seeded in 60-mm dishes and transiently transfected with KIF11 siRNA or control-siRNA. 24h post-transfection, cells were treated with 10 µM cycloheximide (CHX) (Deltaclon S.L.; S7418), an inhibitor of *de novo* protein biosynthesis. At the indicated time points (0, 4, 8, 16 hours), cells were harvested and processed for immunoblotting. Similar procedure was carried out to study AR protein stability after KIF11 overexpression. LNCaP stably transduced cells (pIND_GFP or pIND_KIF11) were seeded in 60-mm dishes plates at a density of $4x10^5$ cells/plate. After 24h of KIF11 expression induction with doxycycline (0.1 µg/ml), cells were treated with 10 µM CHX. At the same timepoints, cells were harvested and protein extracts were prepared for immunoblot analysis. To quantify the change in the rate of AR degradation under the above-mentioned conditions, band intensities of immunoblots were analysed with Image J software, using β-Actin as internal control.

To investigate AR protein degradation through the proteasome pathway upon KIF11 depletion, 22Rv1 ($3.5x10^5$ cells/dish) and C4-2 ($2.5x10^5$ cells/dish) were seeded onto 60-mm plates and transiently transfected with KIF1-siRNA or control-siRNA. 40h post-transfection, cells were treated with 20 µM MG132 (Deltaclon S.L.; S2619), a 26S proteasome complex inhibitor. After 4h treatment, cells were harvested and AR protein levels were analyzed by western blot.

3.10. In vivo experiments

3.10.1. Patient-derived orthotopic xenograft (PDOX)

This experiment was carried out in collaboration with Dr. Aytes and Dr. Villanueva's groups at Institut d'Investigació Biomèdica de Bellvitge's animal core facility (IDIBELL, Barcelona, Spain) under the supervision of Comité Ètic d'Experimentació Animal (CEEA-IDIBELL).

A small fragment of a high risk CRPC patient tumor was implanted orthotopically into the prostate of 7 weeks old male NU-Foxn1nu mice (Harlan) for drug efficacy experiments. Mice were castrated during the same surgery at the animal core facility to deplete serum testosterone levels. 25 days after implantation, mice with homogeneous and palpable tumors were randomized into two groups and treated with vehicle (n=5, p.o) and 4SC-205 (n=8, 40 mg/kg, p.o), three times per week during three weeks. Animal weight was monitored throughout the experiment. At end point, mice were euthanized and tumors were collected and weighed. Each tumor was split in two fragments, one half was fixed in 10% formalin and paraffin embedded for immunohistochemical analysis. The other half was snap frozen in liquid nitrogen and stored for later protein extraction, as explained in section 3.5.1.

3.10.2 Genetically engineered mouse (GEM) xenograft

This procedure was approved by the Ethical Committee of Vall d'Hebron Research Institute (VHIR, Barcelona, Spain) (protocol number 07/18). The NPp53 transgenic model (PTEN and TP53 null) was a kindly gift from Dr. Aytes and had been previously published and described [373].

For *in vivo* analysis of drug combination treatment efficacy, $3x10^6$ NPp53 derived cells were mixed with Matrigel (1:1) and injected subcutaneously into the flank of 7 weeks-old male Nude NMRI mice (Envigo). One week prior to cell injection, mice were castrated by bilateral orchiectomy in order to deplete androgen levels. When tumors reached 150 mm^3 on average, animals were randomly distributed in 4 experimental groups (n=7/group) and treated as follow: vehicle (1% carboxymethylcellulose, p.o), enzalutamide (10 mg/kg, p.o), 4SC-205 (20 mg/kg, p.o) or enzalutamide (10 mg/kg, p.o) + 4SC-205 (20 mg/kg, p.o). Tumors were measured every 2-3 days with an electronic caliper and volumes were estimated based on their width (W) and length (L) according to the formula [$V = (L \times W^2)/2$]. At the end of the experiment, mice were euthanized and tumors were removed, weighed, and cut longitudinally in two fragments. One piece was fixed in 10% formalin and paraffin embedded for immunohistochemical analysis. The other fragment was cut into smaller

pieces, which were snap frozen in liquid nitrogen and stored for later protein and mRNA extraction.

3.11. Statistical analysis

Statistical analyses in this study were performed using GraphPad Prism 8.0 (GraphPad Software, Inc). Statistical differences between two groups were calculated by two-tailed unpaired Student's t-test. Correlation analysis was performed using Pearson's test. Survival information was verified by Kaplan-Meier analysis and p-values were estimated using long-rank test. Half-maximal inhibitory concentrations of drugs (IC_{50}) were determined by non-linear regression approximation. Unless otherwise indicated, values are mean ± SEM of three independent experiments. P values were considered significant as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

4. RESULTS

4.1. Quantitative proteomic analysis reveals M-phase Cell-Cycle proteins upregulated in an in vitro model of androgen independence

The development of PCa from an androgen dependent state to an androgen independent one marks a lethal step in its progression, leading to CRPC. During this evolution, it has been described that a transcriptomic reprogramming characterized by the upregulation of M-phase cell-cycle genes is taking place [192]. Considering that proteins are the final effectors of cell functions, and with the aim to identity new drivers and targets for this lethal disease, in previous work from our group it was explored for the first time if this reprogramming was translated at the protein level (manuscript in preparation). To do so, the proteome of the androgen dependent (AD or castration sensitive) PCa cell line LNCaP was compared with the one from its androgen independent (AI or castration resistant) derivative, LNCaP AI, (for more information see section 3.3.1) using high-throughput quantitative proteomics analysis (**Figure 18**A). From this analysis, a list of differentially expressed proteins between both cell lines was obtained. Since mitotic regulators were described to be overexpressed in CRPC, the upregulated proteins in LNCaP AI cell line were selected for further study. Gene ontology (GO) analysis of these proteins showed that two of the most enriched biological processes were “cell cycle” and “mitosis” (**Figure 18**B), confirming that mitotic regulators may play a key role in CRPC development. Among the most promising candidates were several mitotic kinesins and kinases, some of which have been studied in our laboratory, and some others have already been related with CRPC [286,374,375], giving robustness to our approach. In particular, the present project is focused on the mitotic kinesin KIF11, which protein level was three-fold higher in the androgen independent cell line than in the parental one (**Figure 18**C). This work aimed to clarify the role of KIF11 as a possible driver during the development of CRPC and its therapeutic potential against this lethal disease through pharmacological inhibition.

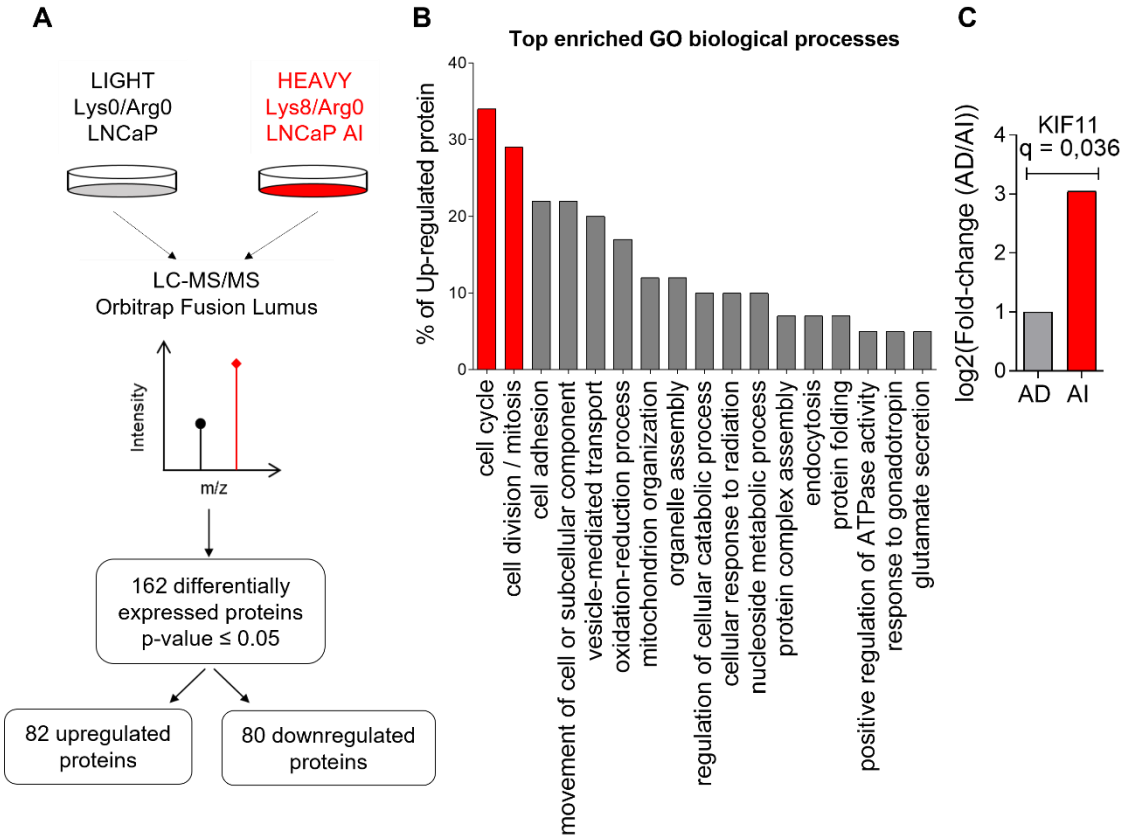

Figure 18. M-phase Cell-Cycle proteins were found upregulated in an in vitro model of androgen independence. (A) Schematic representation of the workflow for the proteomic analysis of LNCaP AD *vs* AI carried out in a previous work and from where we obtained KIF11 as an interesting candidate. **(B)** Enriched GO biological processes of the upregulated proteins in LNCaP AI cell line. **(C)** KIF11 fold-change and q-value obtained in the proteomic analysis.

4.2. Validation of KIF11 as a relevant mitotic candidate in the progression of PCa to androgen independence

4.2.1. KIF11 is overexpressed in androgen independent PCa cell lines and promotes androgen independent PCa cell growth

To confirm the aforementioned data and to further study the association between KIF11 expression and androgen independence, we analysed KIF11 mRNA and protein levels in several representative PCa castration sensitive (LNCaP) and resistant (LNCaP AI, 22Rv1 and C4-2) cell models. As shown in **Figure 19**, all three castration resistant cell lines displayed higher mRNA (**Figure 19**A) and protein expression (**Figure 19**B) than the castration sensitive one, validating the previous results and the potential role of this protein in CRPC development. Moreover, the characterization of KIF11 expression in these cell

lines helped us to select the most appropriate models to perform the subsequent functional assays.

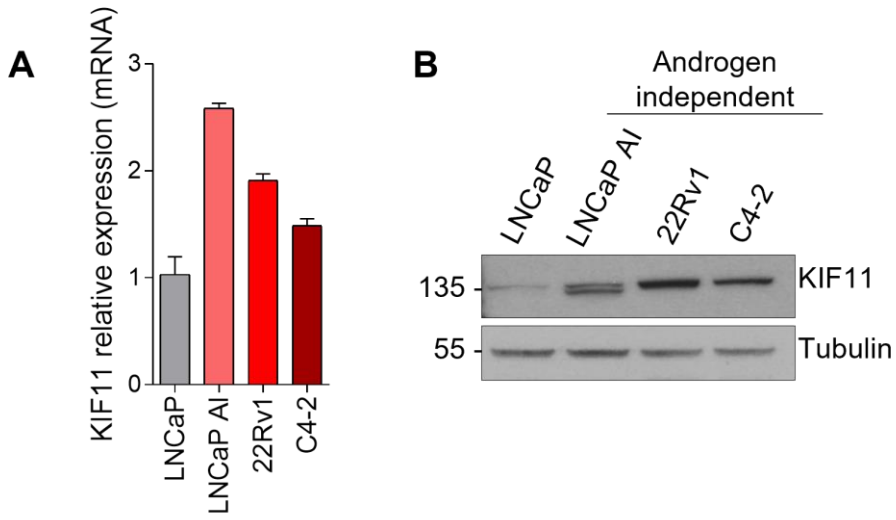

Figure 19. KIF11 transcript and protein levels are upregulated in androgen independent PCa cell models. (A) KIF11 mRNA and **(B)** protein levels in LNCaP, LNCaP AI, 22Rv1 and C4-2 cell lines.

To further investigate the role of KIF11 in the progression to castration resistance, we generated a cell line stably overexpressing KIF11 (pINDUCER20-KIF11) or GFP as control (pINDUCER20-GFP). We used LNCaP cells that, as commented before, are androgen dependent and express low endogenous levels of KIF11, compared with the androgen independent models (see **Figure 19**B). Overexpression was induced with doxycycline and increased KIF11 protein levels were confirmed by western blot (**Figure 20**A). After that, cells were seeded in androgen depleted medium and proliferation was monitored during 4 weeks. Interestingly, we observed that KIF11 overexpression conferred to LNCaP cells higher ability to grow in androgen depleted conditions compared to control cells (**Figure 20**B). This suggests that high levels of this protein conferred androgen independent characteristics and therefore, might be contributing to the development of castration resistance.

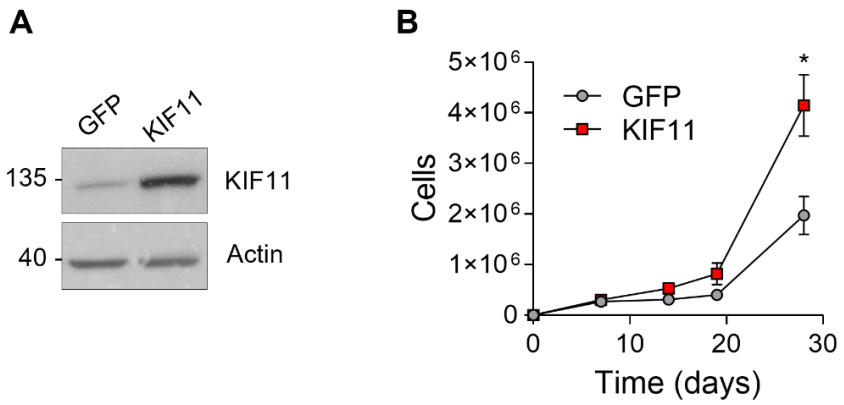

Figure 20. KIF11 overexpression confers androgen independent features to PCa cell lines. (A) Western blot analysis of KIF11 protein levels after gene expression induction in LNCaP stable cell line with 0.1 µg/ml of doxycycline. **(B)** Proliferation assay comparing growth in androgen depleted conditions of LNCaP cells carrying control (GFP) or KIF11 inducible vector. Data shown are mean ±SEM of three independent experiments. *p<0.05 two-tailed Student's t-test.

4.2.2. KIF11 expression is increased in CRPC and is associated with poor prognosis, worse clinico-pathological characteristics and hormonal therapy resistance

To study if this relation between KIF11 overexpression and androgen independence is clinically relevant, we performed a computational analysis using PCa patient public databases to evaluate KIF11 expression at different stages of PCa progression, and more specifically in CRPC. Consistent with the previous findings, the analysis revealed that KIF11 expression is significantly higher in metastatic CRPC (mCRPC) compared with localised PCa and benign prostate tissue in four independent data sets (**Figure 21**A-D). Moreover, KIF11 expression was found significantly higher even when comparing mCRPC with castration sensitive metastatic disease (**Figure 21**D**)**.

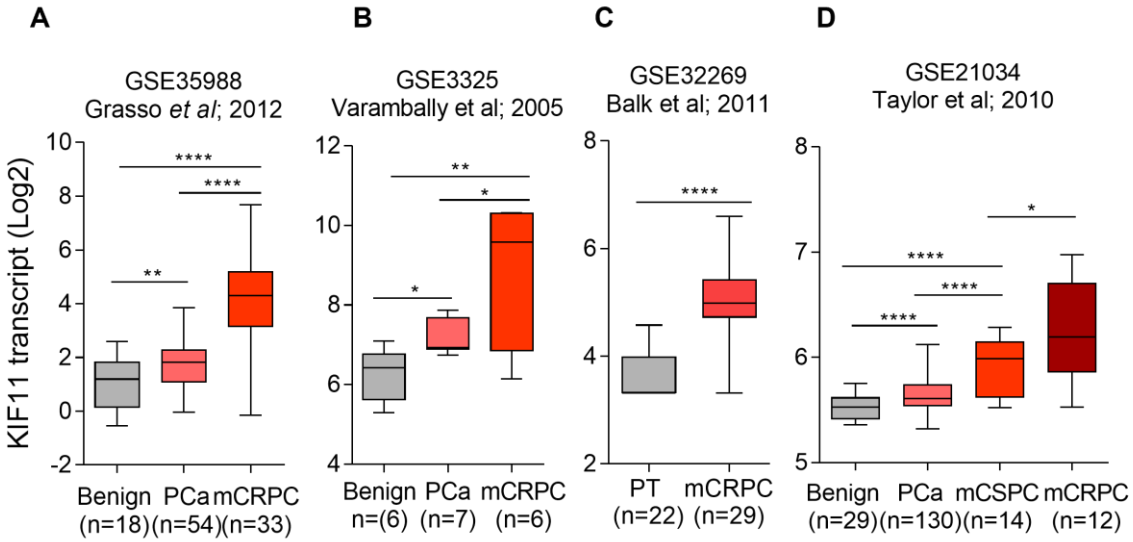

Figure 21. KIF11 expression analysis at different stages of the disease. KIF11 expression was examined using 4 independent data sets comparing **(A,B)** normal prostate, localised disease (PCa) and mCRPC (Grasso and Varambally datasets); **(C)** localised PCa with mCRPC (Balk dataset) and **(D)** normal prostate, localised disease, mCSPC and mCRPC (Taylor dataset). Sample size (n) is specified for each group under the graphs. Transcription data was retrieved using Geo2R platform. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-tailed Student's t-test.

We then studied a possible relation between KIF11 expression and other clinico-pathological characteristics. We found that high KIF11 transcriptional levels were related with higher Gleason score, a histopathological indicator of PCa aggressiveness and prognosis (**Figure 22**A). Patients with higher Gleason values usually display more aggressive disease and worse prognosis (for more information see section 1.2.4.2). In addition, increased levels of the motor protein were significantly associated with BCR, suggesting a potential role of KIF11 as a prognostic indicator of tumor relapse (**Figure 22**B). In line with that, patients with high KIF11 expression displayed worse disease and progression free survival (**Figure 22**C and D), also indicating that high KIF11 levels are associated with more aggressive forms of the disease.

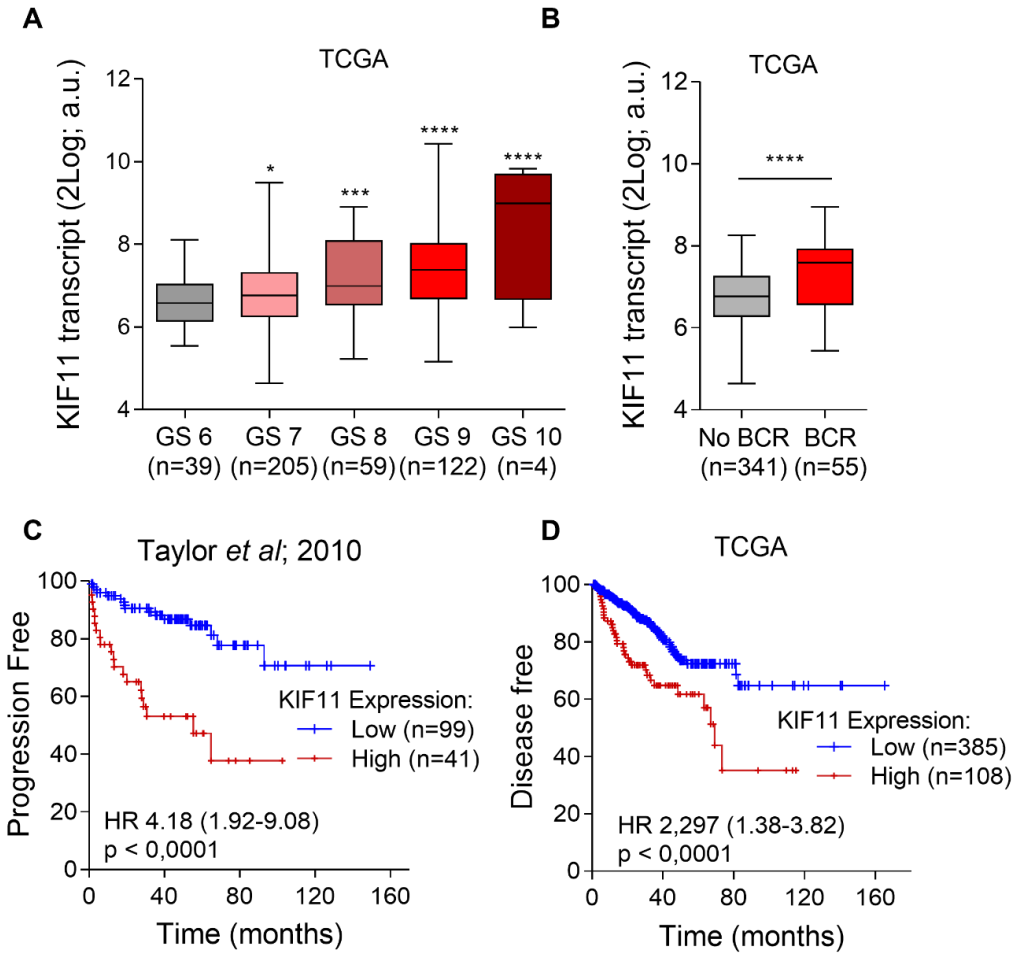

Figure 22. KIF11 expression is associated with higher Gleason scores, biochemical recurrence, and poor prognosis. (A) TCGA dataset analysis of KIF11 expression in PCa cases with different Gleason scores (GS) and **(B)** with or without biochemical recurrence (BCR). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-tailed Student's t-test. **(C, D)** Kaplan-Meier curves from univariate analysis. P-values were estimated with log-rank test. High levels of KIF11 are significantly related with **(C)** poor disease and **(D)** progression free survival.

In order to validate the results found in the *in silico* analysis, we studied KIF11 mRNA and protein levels in PCa human tissue samples obtained from Vall d'Hebron Hospital (Barcelona, Spain) and from the Pathological Anatomy Unit of the Marche Polytechnic University (UNIVPM, Ancona, Italy), by RT-qPCR and immunohistochemistry (IHC), respectively (patient characteristics are specified on and **Table 9** and **Table 10**).

For mRNA analysis, KIF11 expression in FFPE tissue samples from patients with PCa was compared with the expression of adjacent normal tissue. Samples were obtained after tumor removal by radical prostatectomy. Tumoral areas were laser microdisected in order to obtain material exclusively from cancer cells. As it can be observed in **Figure 23**A, KIF11 expression was significantly higher in tumoral samples compared with healthy tissue. Moreover, as patients were followed-up during more than ten years after surgery, we were

able to subdivide the patients in two groups, those who developed metastasis during that period and those who did not. We compared KIF11 expression between these groups and found that primary tumors from patients who eventually developed metastasis displayed higher KIF11 expression than those who did not (**Figure 23**B), in line with previous observed results.

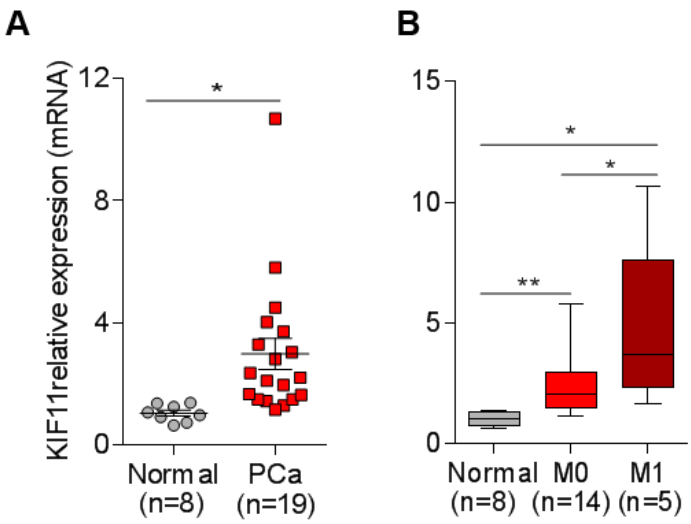

Figure 23. KIF11 is overexpressed in primary tumors from patients who develop metastatic disease. (A) KIF11 transcript in levels in normal *vs.* tumoral tissue from PCa patients. **(B)** KIF11 mRNA expression in normal prostate tissue and in samples from patients who developed (M1) or not (M0) metastatic disease. *p<0.05, **p<0.01, two-tailed Student's t-test.

In relation with protein expression, we analysed KIF11 levels in two sets of samples. The first was comprised by benign conditions (BPH, n=8), PCa-naïve (N=8) and CRPC (N=8) FFPE patient samples. The other one included biopsy samples from patients diagnosed with already advanced disease that performed two different outcomes in response to therapy. Those that still responded to ADT after 24 months of treatment were categorised as "Responders" (n=9). Patients that developed CRPC before one year of treatment were classified as "Resistant" (n=9). Although the number of samples per group is not very high, it is important to consider that CRPC samples are difficult to obtain, as those patients rarely undergo a second surgery after treatment initiation [376]. Also, patients presenting with advanced disease at the time of diagnosis are not very common [27].

To quantify protein expression, each stained sample was scored using H-score system, in which values from 0 to 3 were assigned to cells depending on their stain intensity, being 0 no staining and 3 the maximum grade of intensity (for more detail see section 3.5.4). Accordingly with previous results, we found that CRPC samples displayed increased KIF11 protein levels compared to both, PCa treatment-naïve tissues and benign conditions (BPH)

(**Figure 24**A-C). Moreover, only cells from CRPC samples displayed intensity values of 3 (**Figure 24**B). Likewise, higher KIF11 protein expression was observed in biopsy samples from patients with advanced disease that became resistant to ADT before one year of treatment compared to those from patients that still responded after 24 months (**Figure 24**D-E). Again, highest intensity values were only detected in samples from resistant patients. (**Figure 24**E). Once more, these results indicate that KIF11 might be participating in development of CRPC and hormone-therapy resistance. Moreover, these findings suggest that KIF11 could be useful as a biomarker of prognosis and response to therapy. However, although promising, the results should be verified in a bigger cohort of patients.

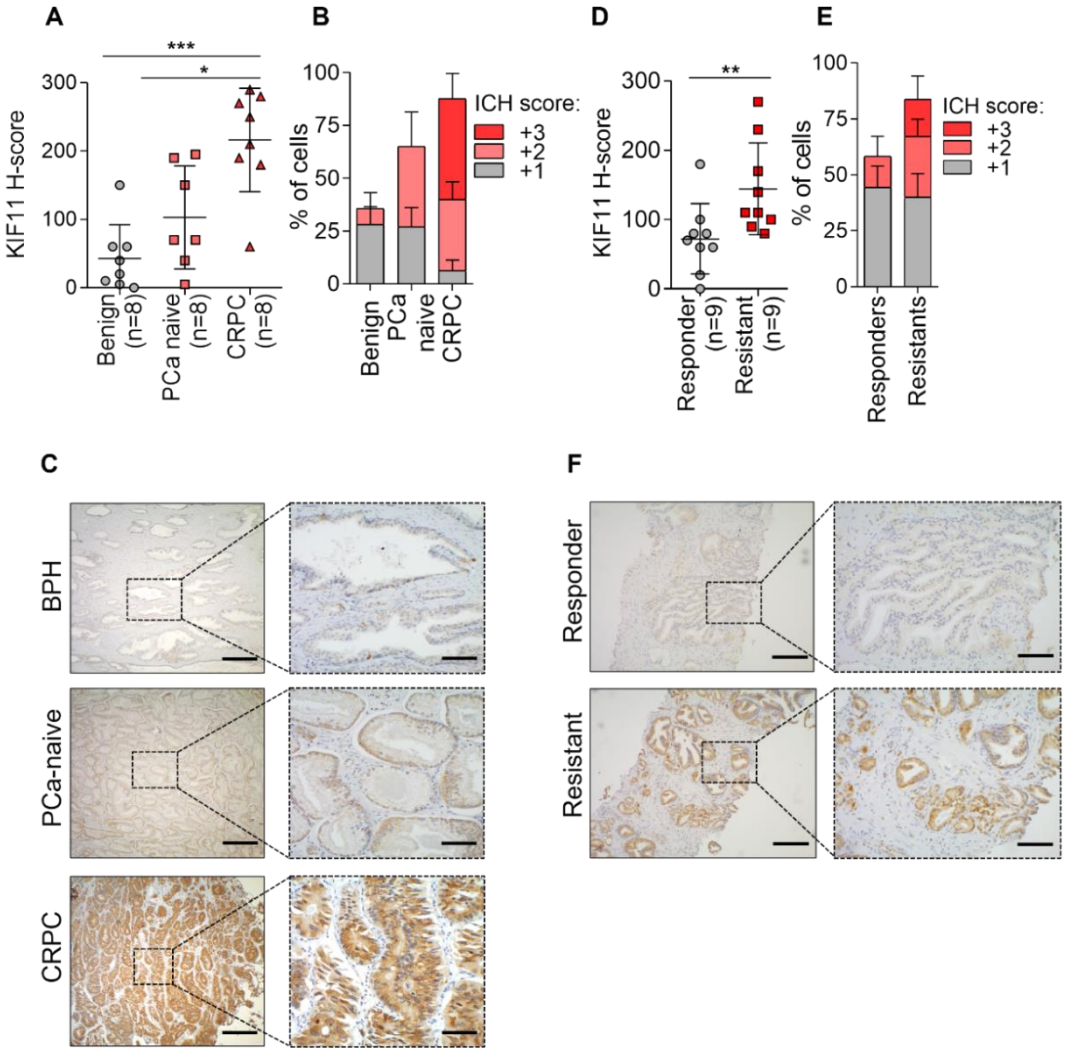

Figure 24. KIF11 protein expression is higher in CRPC and hormonal therapy resistant patients. (A, D) KIF11 H-score in **(A)** benign conditions, treatment-naïve and CRPC tumor tissues, and in **(D)** tissue samples from patients with advanced disease that were still sensitive to ADT after 24 months (responders) or became resistant before one year of treatment (resistant). **(B, D)** Average percentage of cells displaying one of the different staining intensities in each of the indicated groups of samples. **(C, F)** Representative images from (A) and (B), respectively. Scale bars, 50 µm (C left) and 100 µm (F left). Inset scale bars, 25 µm. * p<0.05, ** p<0.01, ***p<0.001 two-tailed Student's t-test.

Given that KIF11 is correlated with poor prognosis, more aggressive forms of the disease, and seems to be associated with ADT resistance, we also wanted to study a possible implication of this protein with the development of resistance to other hormonal-therapies such as enzalutamide. This agent is a potent second-generation AR inhibitor commonly used for the treatment of patients who develop resistance to ADT and therefore CRPC. For that aim, we used two independent databases containing transcriptional information of enzalutamide resistant PCa models to analyse KIF11 expression. We could observed that, although not significant, a clear tendency of higher KIF11 expression was performed by C4-2 cell lines (**Figure 25**A) and LNCaP xenograft models (**Figure 25**B) that were resistant to the AR inhibitor compared to their controls. Moreover, we subjected the CRPC cell line C4-2 to prolonged treatment with enzalutamide at a constant concentration of 10 µM for 60 days to study its effects on KIF11 levels. KIF11 mRNA (**Figure 25**C) and protein expression (**Figure 25**D) were monitored during the 2 months of treatment. We observed an initial decreased in KIF11 levels after the first week of treatment, which started to increase after 15 days and continued raising for two months. These observation supports that prolonged treatment with the AR inhibitor enzalutamide increases KIF11 expression, and this fact could be contributing to the development of resistance.

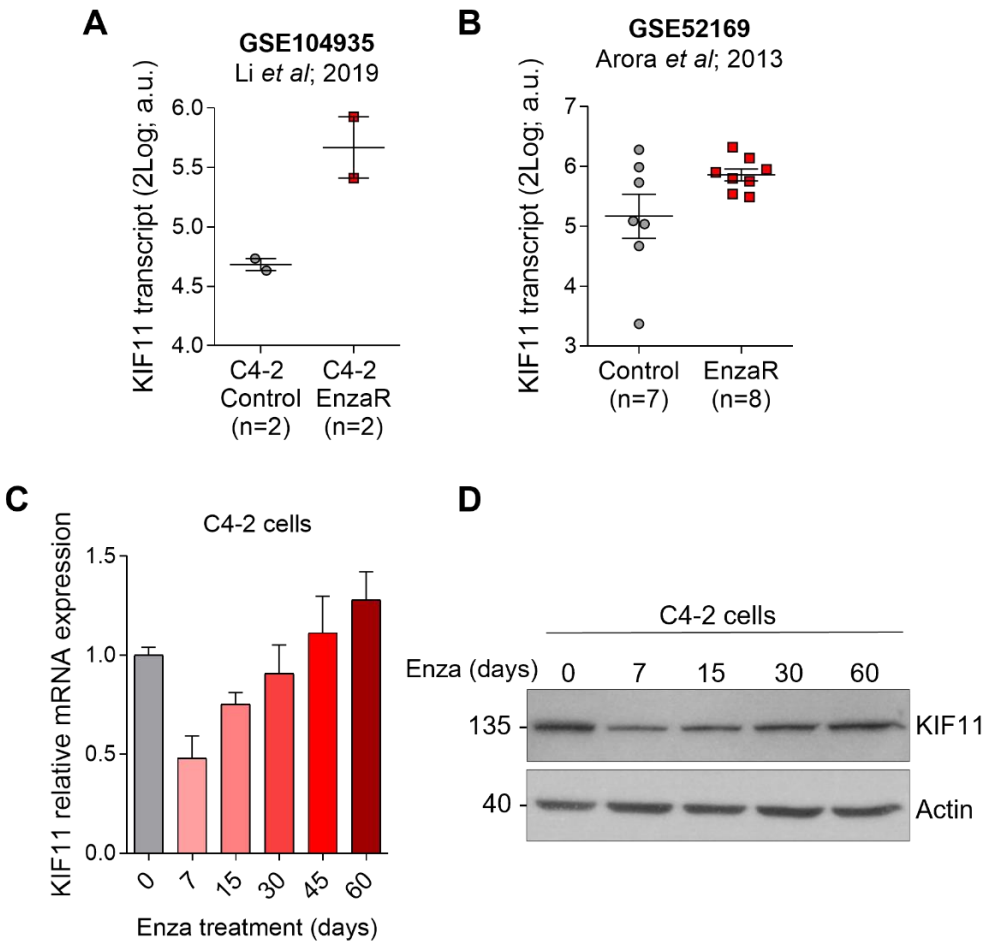

Figure 25. Time dependent upregulation of KIF11 by enzalutamide. KIF11 expression was analysed in two independent datasets of **(A)** C4-2 cells sensitive and enzalutamide resistant (EnzaR) and **(B)** control and enzalutamide resistant LNCaP mouse xenograft tumors. **(C, D)** C4-2 cells were treated with enzalutamide (10 µM) during 60 days. **(C)** mRNA levels were analysed by RT-qPCR and **(D)** protein levels by immunoblot at the indicated time points since treatment initiation. Data shown are mean ±SEM.

All things considered, the analysis of KIF11 protein and mRNA expression levels from multiple cell lines, patient datasets and tissue samples indicated that KIF11 might play an important role during PCa progression, in particular to CRPC, and in the development of therapy resistance.

4.3. KIF11 inhibition with 4SC-205 induces mitotic arrest and apoptosis in PCa cells

As all the above-described evidence indicates a possible important role of KIF11 in the progression of PCa, we proceeded to study the effect of KIF11 pharmacological inhibition in PCa cell models. It is well stablished that KIF11 inhibition results in an accumulation of

cells in mitosis exhibiting a characteristic monopolar spindle phenotype, which eventually leads to cell death by apoptosis [316]. Based on that, we wanted to find out if the novel KIF11 small-molecule inhibitor 4SC-205 was able to reproduce these effects in PCa models, as we have previously demonstrated it for neuroblastoma cells in a collaboration project with Dr. Miquel Segura's group (Translational Research in Child and Adolescent Cancer Laboratory, Vall d'Hebron Research Institute, Barcelona, Spain) [368].

To do so, we evaluated the mitotic and apoptotic index after 4SC-205 treatment in the universally used PCa cell models LNCaP and Du145. Cells were treated with the inhibitor and after 12, 24 and 48h of drug exposure were simultaneously stained with Hoechst dye and propidium iodide (PI) for fluorescence microscopy analysis. The mitotic index was determine using Hoechst staining, counting the number of cells with condensed chromosomes over the total. This analysis revealed a significant increase in mitotic cells after 12h of drug exposure, indicating the foreseeable mitotic arrest. The number of mitotic cells started to decrease after 24h-48h of treatment, most likely due to cell death, with slight differences between Du145 (**Figure 26**A and B) and LNCaP cells (**Figure 26**C and D).

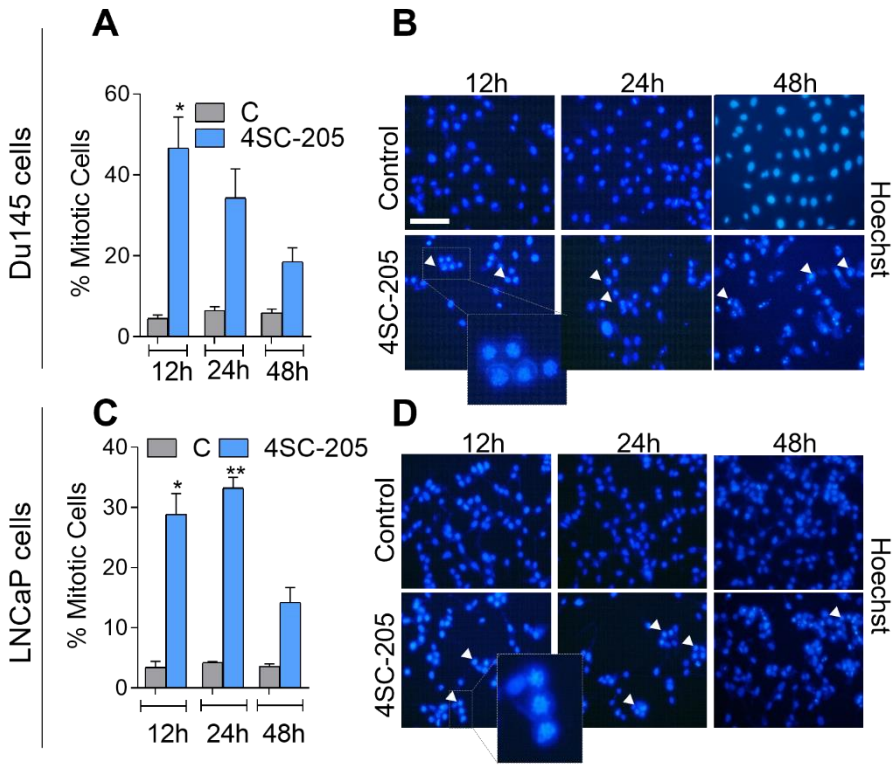

Figure 26. KIF11 inhibition with 4SC-205 induces a mitotic arrest in PCa cells. (A, C) Mitotic index assay quantification in **(A)** Du145 and **(C)** LNCaP cell lines treated with KIF11 inhibitor (25 and 6 ηM, respectively) for the indicated times and quantified by analysis of condensed chromosomes with Hoechst staining. **(B, D)** Representative images from (A) and (C), respectively. Arrowheads point at nuclei with condensed chromatin. Scale bar, 50 μm. Data are mean of two independent experiments ±SEM. *p<0.05, **p<0.01 two-tailed Student's t-test.

For cell death analysis, we evaluated the number of cells with positive PI staining over the total number of cells, as this dye cannot enter the plasma membrane of living cells [371]. As expected, we observed an oppositive tendency than the one observed for the mitotic index in both cell models. Initially, the proportion of positive PI stained cells is small and it significantly increased after 24h of drug exposure in both cell lines (**Figure 27**), suggesting that the mitotic arrested cells eventually suffer cell death.

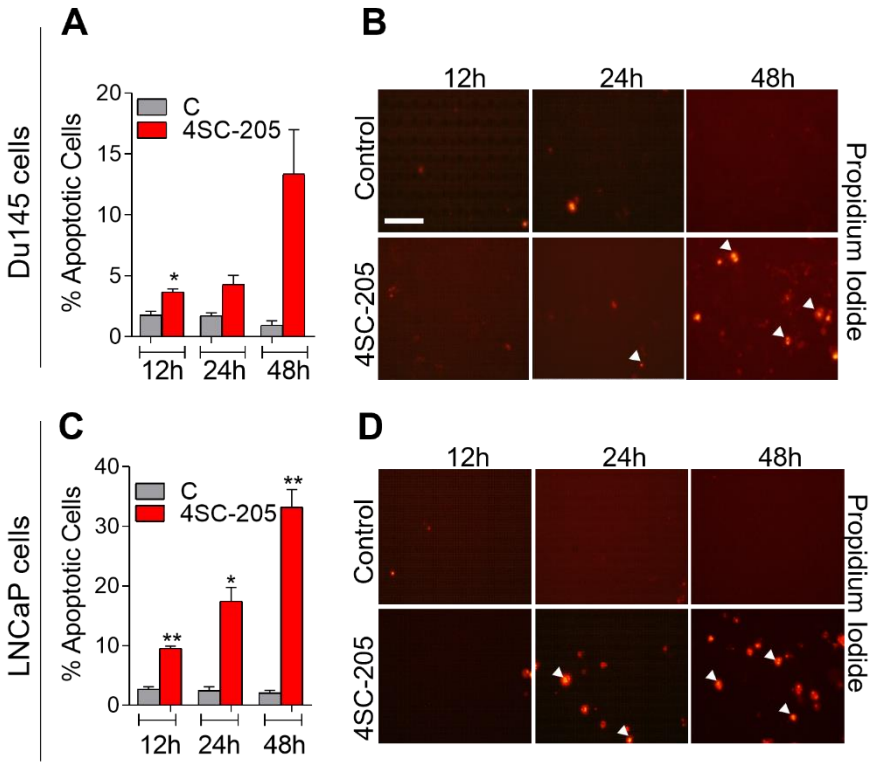

Figure 27. KIF11 pharmacological inhibition with 4SC-205 induces cell death in PCa cells. (A, C) Cell death assay quantification in **(A)** Du145 and **(C)** LNCaP cell lines treated with 4SC-205 (25 and 6 ηM, respectively) for the indicated times and quantified by analysis of propidium iodide-stained cells. **(B, D)** Representative images from (A) and (C). Arrowheads point to death cells. Scale bar, 50 μm. Data are mean of two independent experiments ±SEM. *p<0.05, **p<0.01 two-tailed Student's t-test.

Consistent with the previous results, immunoblot analysis of protein extracts from 4SC-205 treated cells showed accumulation of Cyclin B1 and increased phosphorylation of Histone 3 (H3) at serine 10 compared to vehicle (**Figure 28**A and C), both typical markers of mitotic arrest [377,378]. Also, increased cleavage of caspase-3 and of its substrate PARP was detected (**Figure 28**B and D), confirming an activation of the apoptotic signalling pathway upon 4SC-205 exposure. These results indicate that 4SC-205 is able to induce cell cycle arrest during mitosis, which eventually leads to cell death by apoptosis in PCa cell lines.

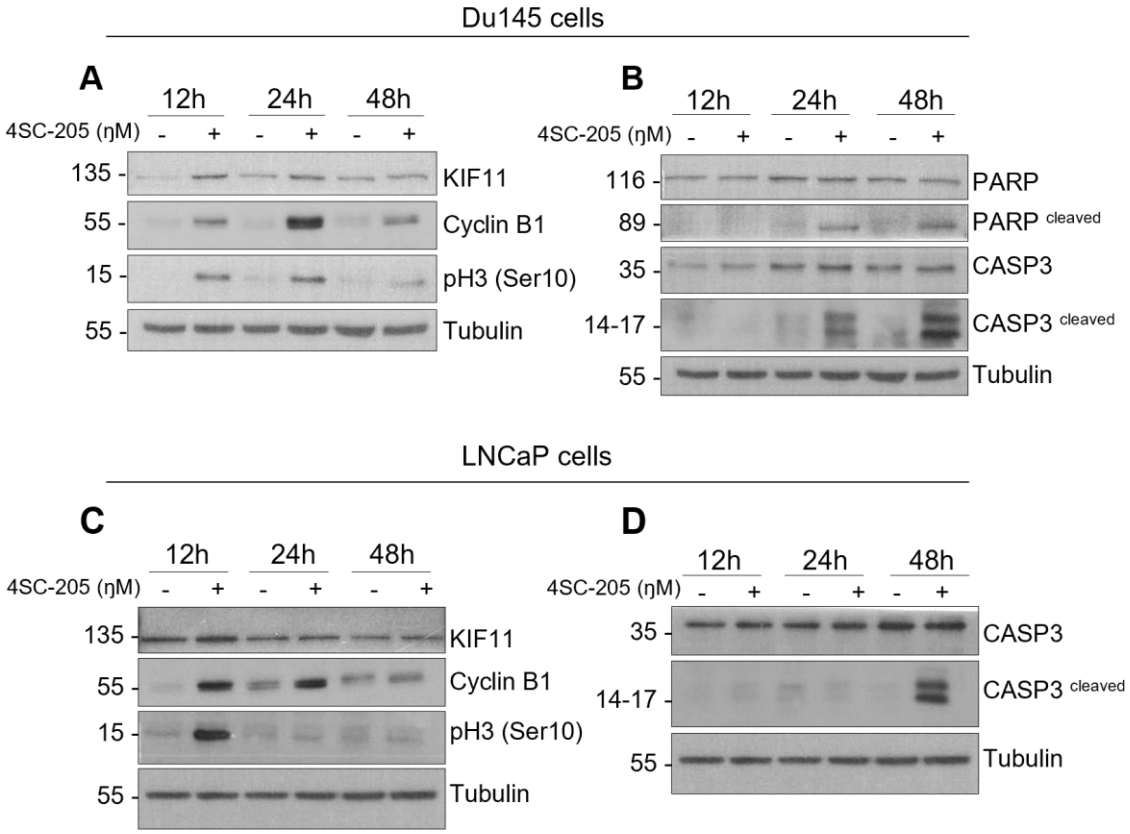

Figure 28. 4SC-205 induces a mitotic arrest that eventually leads to cell death via apoptosis in PCa cell lines. (A, C) Western blot analysis of the mitotic proteins Cyclin B and phospho-histone H3 at serine10 in **(A)** Du145 and **(C)** LNCaP cells after 4SC-205 treatment (25 and 6 ηM, respectively) for the indicated times to confirm the mitotic arrest. **(B, D)** Western blot analysis of the indicated apoptosis-related proteins in **(B)** Du145 and **(D)** LNCaP cells after 4SC-205 treatment (25 and 6 ηM, respectively) for the indicated times to verify the activation the apoptosis signalling pathway.

To further verify the above results, we decided to perform cell cycle analysis by flow cytometry after 4SC-205 treatment on Du145 cells. To do that, cells were previously treated with thymidine for 24h and then released, to synchronize them at G1/S phase. Then, cells were treated with the inhibitor and after 12 and 24h of drug exposure, cell cycle profile was analyzed by flow cytometry-mediated analysis of DNA content. **Figure 29**A). As it can be seen in **Figure 29**B and C, the fraction of cells arrested in G2/M phase increased remarkably upon 4SC-205 treatment at both times. Moreover, to test if the observed effects caused by 4SC-205 can be reversed, cells were exposed to the inhibitor but this time, cells were wash out and medium was refreshed after 12h treatment. Following 12h of inhibitor release, cell cycle was analyzed again by flow cytometry. We could observe that after the removal of the inhibitor, the percentage of cells in each phase of the cell cycle came back to a normal ratio (**Figure 29**C), comparable to vehicle treated cells. This fact becomes especially important when deciding the administration schedule of a specific agent. In this

particular case, in which the effect of the inhibitor is reversible, a more constant dosing might be needed in order to achieve higher antitumor efficacy.

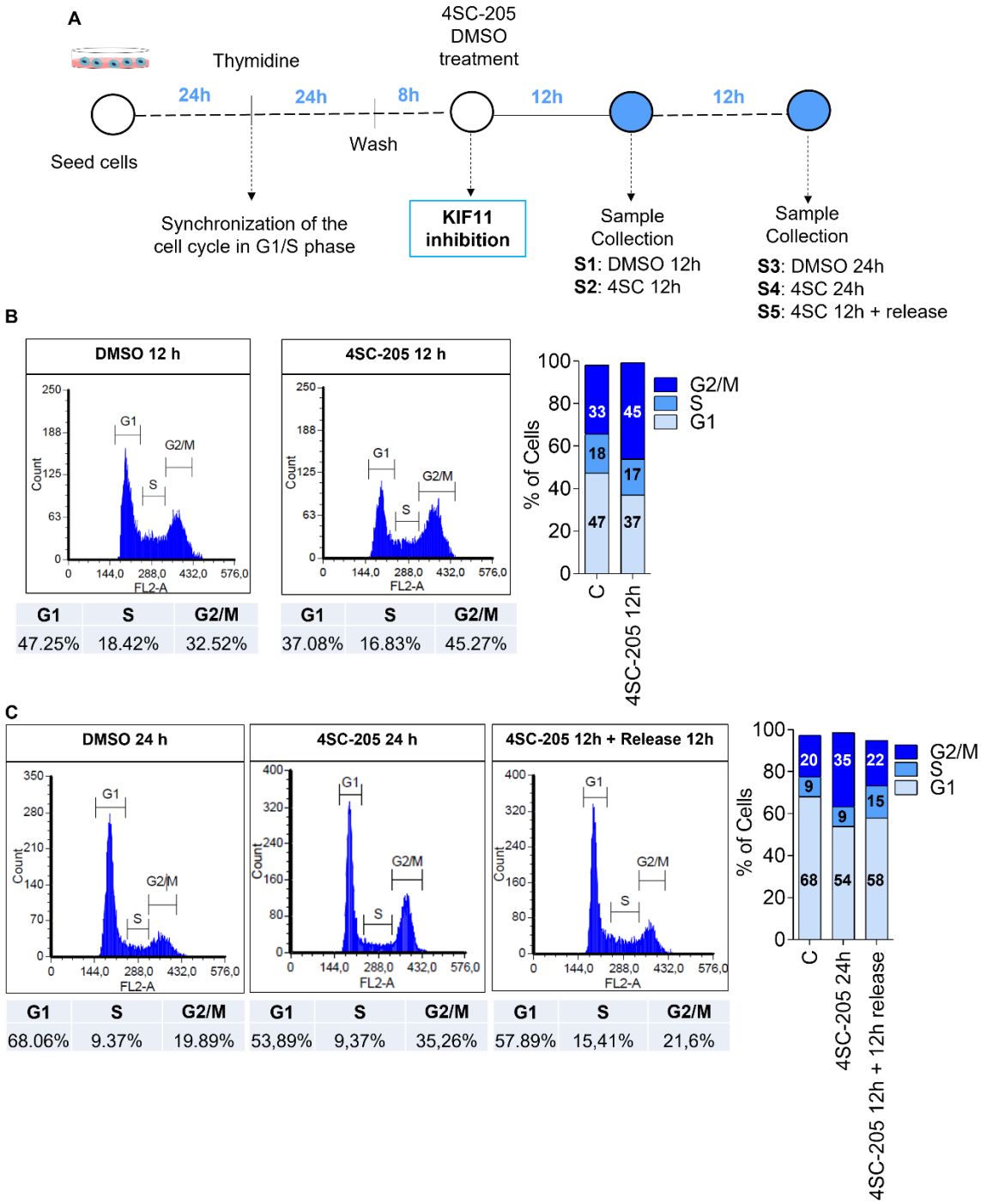

Figure 29. 4SC-205 induces a mitotic arrest in a reversible manner. **(A)** Schematic representation of the experimental procedure. **(B)** Cell cycle profile of Du145 analysed by flow cytometry after 12h of vehicle or 4SC-205 treatment. **(D, E)** Cell cycle analysis of Du145 cells after, from left to right, 24h of treatment with vehicle, the inhibitor, and after 12h of 4SC-205 treatment plus 12h release.

4.4. KIF11 pharmacological inhibition with 4SC-205 effectively impairs PCa viability *in vitro* and *ex vivo*

4.4.1. 4SC-205 impairs viability and clonogenicity of PCa cell lines *in vitro*

Several KIF11 inhibitors are currently being tested in preclinical studies [379,380], however the efficacy of 4SC-205 in PCa models has not been reported. Having shown that KIF11 inhibition by 4SC-205 recapitulates the classical phenotype of KIF11 knockdown/inhibition in PCa cells, resulting in a mitotic arrest followed by apoptosis, we ought to investigate the sensitivity of PCa cell lines to KIF11 inhibition with this agent. For that aimed, we used a panel of PCa cell lines, for which KIF11 expression was previously verified (**Figure 30**A and B). We observed that all cells displayed a similar sensitivity to KIF11 inhibition by 4SC-205 treatment, (**Figure 30**C) indicating that the inhibitor may be effective in a broad spectrum of PCa tumors, which is especially important given the heterogeneous nature of the disease. Moreover, it is noteworthy that RWPE-1, a non-tumoral epithelial derived prostate cell line was by far the less affected by the inhibitor, suggesting that KIF11 is a good candidate to specifically target dividing malignant cells. Furthermore, colony formation assays also showed a reduced colony formation capacity upon treatment in all cell lines, being again RWPE-1 the less affected model (**Figure 30**D and E).

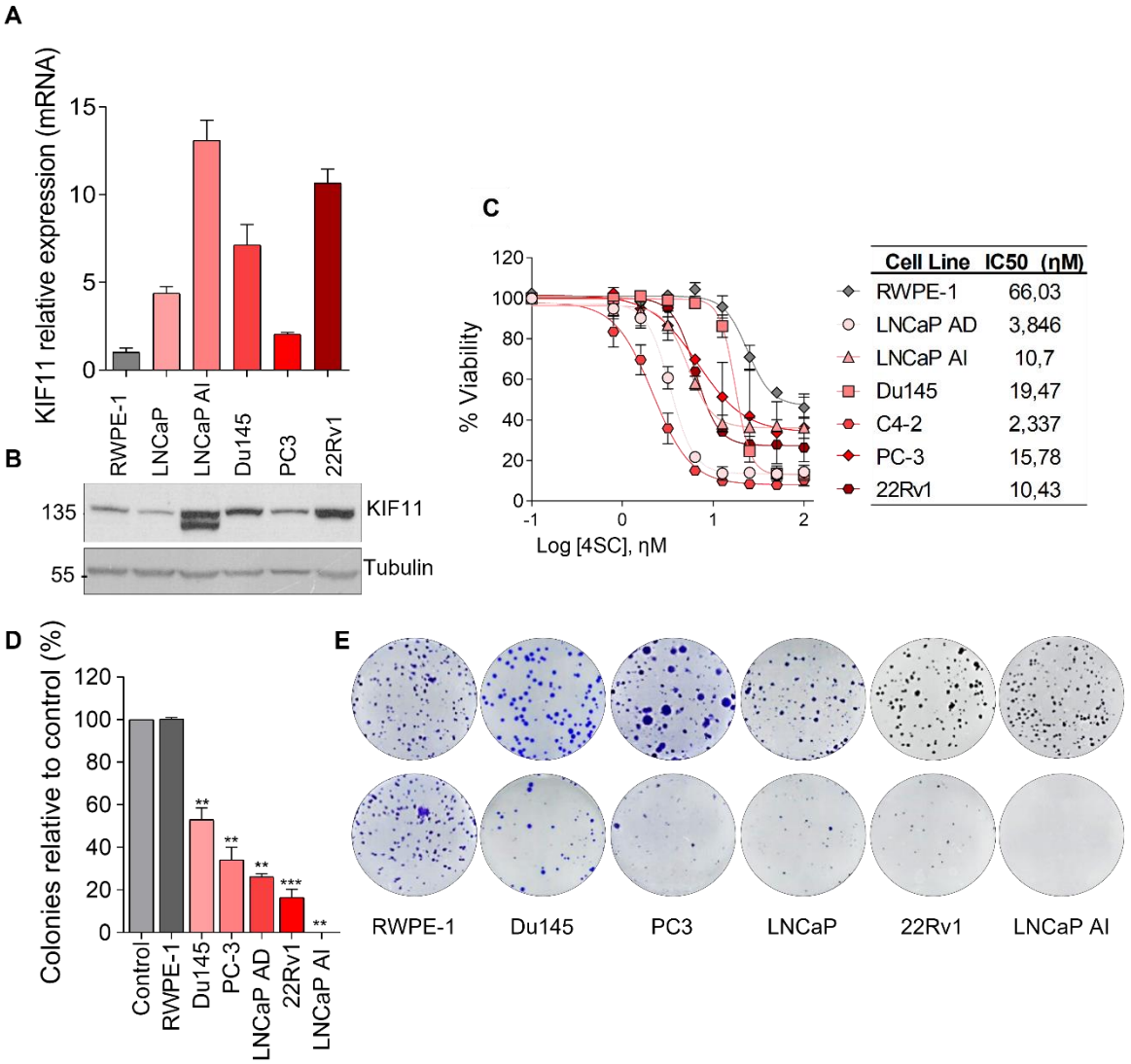

Figure 30. KIF11 pharmacological inhibition reduces cell viability and clonogenic capacities in PCa cell lines in vitro. (A) KIF11 mRNA and **(B)** protein levels in a panel of PCa cell lines. **(C)** Dose-dependent effect on cell viability of the indicated PCa cell lines after 72h treatment with 4SC-205 (left) and the corresponding IC_{50} values (right), calculated as the average of three independent experiments. **(D)** Colony formation assay with the indicated PCa cells treated with 4SC-205 during 10 days **(E)** Representative macroscopic images from (D). Data shown are mean of three independent experiments ±SEM. *p<0.05, **p<0.01, ***p<0.001 two-tailed Student's t-test.

4.4.2. 4SC-205 reduces formation and viability of patient-derived tumor spheroids

To further evaluate the implication of 4SC-205 as an anti-cancer agent, we decided to test its antitumoral activity in three-dimensional (3D) models, which have been shown to reflect more accurately the translational value of *in vitro* drug efficacy studies than two-dimensional cell monolayers [381]. Moreover, in order to increase the clinical relevance, 3D models were generated from PCa patient-derived primary cultures, which were obtained and expanded

as explained in section 3.3.2. Then, these patient-derived cells were cultured in anchorage-independent conditions, to promote self-aggregation of cells forming spheroids (schematic representation shown in **Figure 31**A), which were exposed to increasing concentrations of 4SC-205. All cultures used for this analysis were derived from patients with advanced and aggressive disease (**Table 16**). After 72h of drug exposure, MTS assay was performed to determine cell viability. In all three cases, a significant reduction in patient-derived spheroid formation capacity and viability upon 4SC-205 treatment in a dose dependent manner was observed (**Figure 31B** and **C**). The fact that KIF11 inhibition effects observed in cell monolayers are reproducible in patient-derived 3D models emphasizes its potential as a valid therapeutic target for the treatment of advanced PCa.

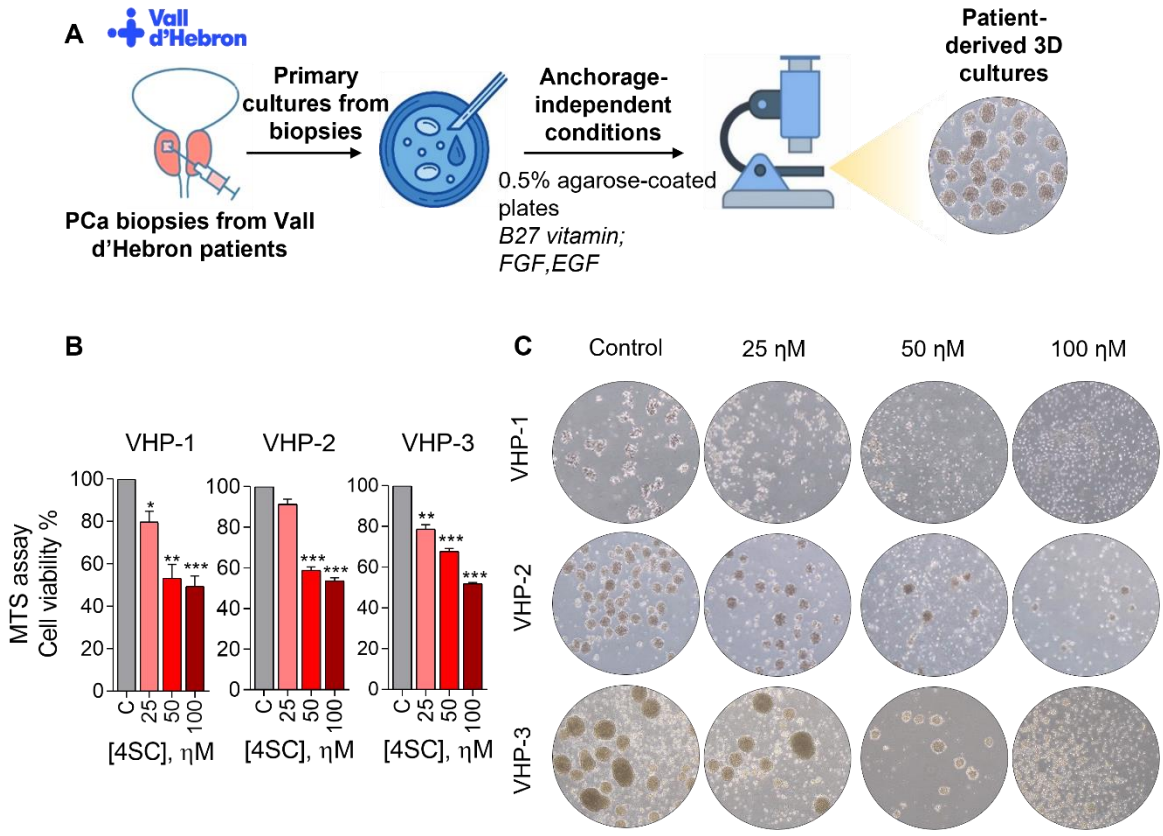

Figure 31. 4SC-205 reduces the viability of PCa tumor spheroids from PCa patient derived cells. (A) Patient-derived tumoral cells were obtained from Vall d'Hebron Hospital patient biopsies and cultured in anchorage independent conditions as shown in the diagram. **(B)** Quantitative MTS cell viability analysis was performed after 72h of drug exposure in the indicated PCa patient-derived spheroids (n=3/condition) * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001 two-tailed Student's t-test, error bars ±SEM **(C)** Representative microscopic images of tumor spheroids.

4.5. 4SC-205 effectively inhibits tumor growth of CRPC patient-derived orthotopic xenograft (PDOX)

Increasing evidence shows that patient-derived xenograft (PDX) models generated by implantation of clinical tumor samples into immunodeficient mice retain the intratumoral heterogeneity and biology of the original tumor and allow to accurately assess the clinical potential of new treatment modalities [382]. In the case of PCa, orthotopic grafting is considered the best approach, as the organ of origin seems to provide important characteristics for the establishment and progression of the disease [383].

In order to evaluate the antitumoral activity of 4SC-205 *in vivo*, a PDOX model was generated from the tumor of a patient with high risk CRPC, by surgical implantation of the specimen into the prostate of the mice. To better reproduced the clinical scenario, mice were castrated during the same surgery. 25 days after tumor implantation, mice with homogeneous and palpable tumors were randomized in two groups and treated three times per week during 3 weeks with 4SC-205 (40 mg/Kg, p.o) or vehicle (**Figure 32**A). At endpoint, tumors were excised, measured, and weighted. 4SC-205 treated mice exhibited a reduction in tumor volume and tumor weight of 7.4 and 5.8-fold respectively, compared to vehicle treated group (**Figure 32**B and C). Furthermore, histopathological (**Figure 32**D and E) and immunoblot analysis (**Figure 32**F and G) revealed that 4SC-205 treated tumors contained a significantly higher fraction of cells with increased H3 phosphorylation compared to vehicle treated ones, addressing a specific and an active effect of the inhibitor after three weeks of treatment. This was further confirmed by an enhanced activation of the apoptotic signalling pathway, proved by the increased in caspase-3 cleavage (**Figure 32**F). Expression of the target protein in the tumors was verified by IHC and immunoblot (**Figure 32**D and F). Animal weight was monitored throughout the experiment with no important differences detected between the two groups (<10%) (**Figure 32**H).

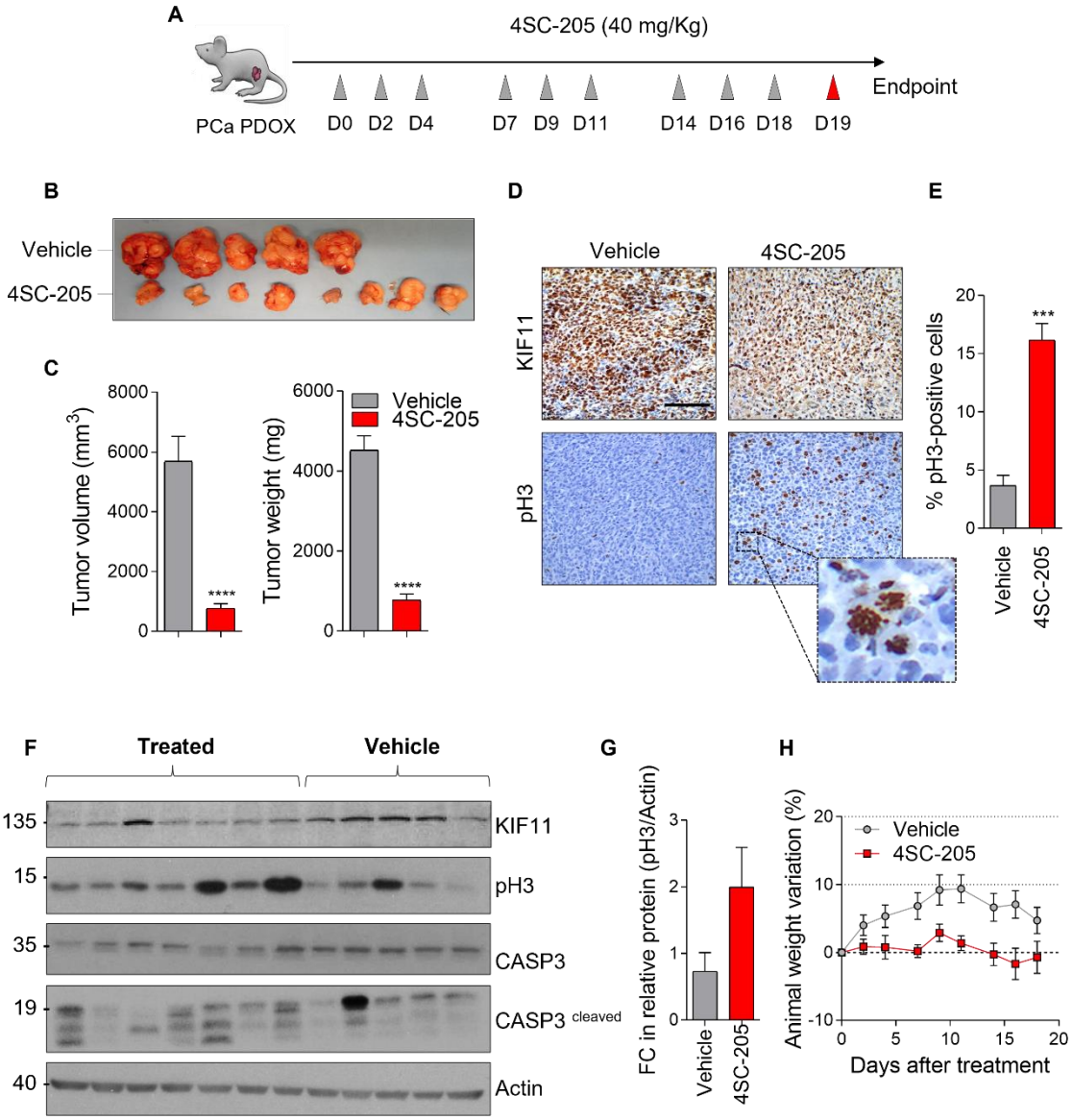

Figure 32. 4SC-205 significantly reduces tumor growth in a CRPC PDOX model. **(A)** Schematic representation of the PDOX treatment schedule. **(B)** Representative macroscopic image of excised tumors at the end of the experiment. **(C)** Average volume (left) and weight (right) of resected tumors from mice treated with 4SC-205 (N=8) or vehicle (N=5) at endpoint. **(D)** IHC staining using anti-KIF11 (top) and anti-pH3 (bottom) antibodies (magnification, x20. Scale bar, 50 µm). **(E)** Quantification of pH3 positive cells in histological sections of PDOX tumors. **(F)** Western blot analysis of the indicated proteins in PDOX excised tumors. **(G)** pH3 protein level quantification from (F) by densitometry analysis. **(H)** Mouse weight variation during three weeks of vehicle or 4SC-205 oral administration. Data are represented as mean ±SEM. ***p<0.001, ****p<0.0001, two-tailed Student's t-test.

4.6. AR and KIF11 expression positively correlates in PCa

The AR is a transcriptional factor that regulates the expression of its target genes, and it has been shown to have crucial roles, not only in normal prostate development, but also in prostate tumorigenesis, even at castration resistant stage [384]. Moreover, the AR was shown to be the responsible of the overexpression of M-phase Cell-Cycle genes/proteins that we and others have observed during CRPC development [192]. Based on that, and on the potential role that KIF11 seems to play in PCa progression, we decided to explore a possible correlation between the expression of KIF11 and the AR. To do so, we performed gene expression correlation analysis using three independent PCa datasets (Sueltman, TCGA, and Dunning), and one of normal prostate tissue (GTEx dataset). Interestingly, we found a positive correlation between KIF11 and AR expression in all three PCa datasets (**Figure 33**A), that was not observed in the normal prostate tissue database (**Figure 33**B). All these made us thinks that maybe a functional interaction between the AR and KIF11 is taking place during PCa progression.

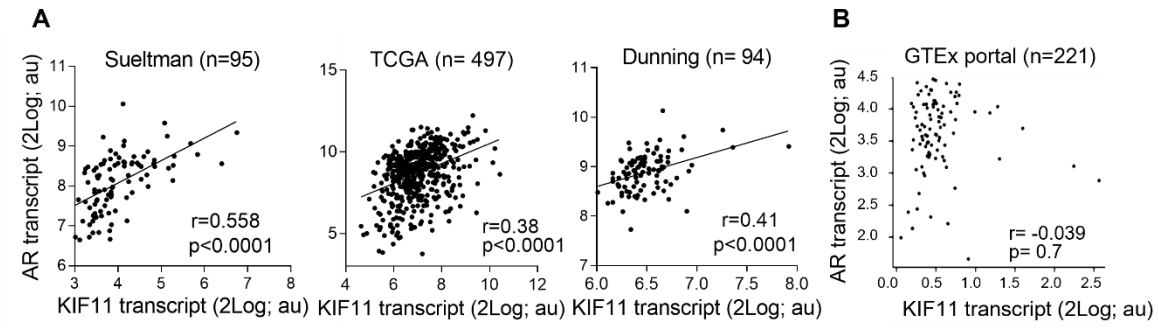

Figure 33. KIF11 expression positively correlates with AR expression in PCa but not in normal prostate tissue. (A) KIF11/AR correlation in three independent PCa datasets. **(B)** Association between KIF11 and AR expression in normal prostate samples from GTEx repository. Correlations were determined by Pearson analysis.

4.7. AR function modulates KIF11 expression in PCa

Considering that (I) the AR acts as a transcriptional factor regulating the expression of its target genes driving PCa progression [384]; (II) that during CRPC development, the AR was described to upregulate a subset of mitotic genes; (III) that KIF11 is an important mitotic protein overexpressed in CRPC and (IV) given the positive correlation that we found between KIF11 and AR expression, we decided to investigate a potential transcriptional regulation of KIF11 expression by the AR in PCa. To explore this possibility, we carried out several functional assays modulating AR activity and monitoring KIF11 expression changes upon that modulation in several PCa *in vitro* models.

Firstly, androgen dependent cell line LNCaP was cultured in androgen depleted medium for 72h before being challenged with DHT, in order to induce AR activity. Interestingly, we found that after 4h and 24h of androgen stimulation with DHT, KIF11 transcript and protein levels significantly increased, respectively (**Figure 34**A and B). To determine whether androgen induction of KIF11 was mediated by the AR, we exposed LNCaP cells to the AR antagonist enzalutamide. We observed a reduction on KIF11 transcript level, which was comparable with the decrease observed in the AR canonical target gene PSA, which expression is commonly used as a biomarker of AR transcriptional activity (**Figure 34**C). This same effect was also observed on KIF11 protein levels (**Figure 34**D).

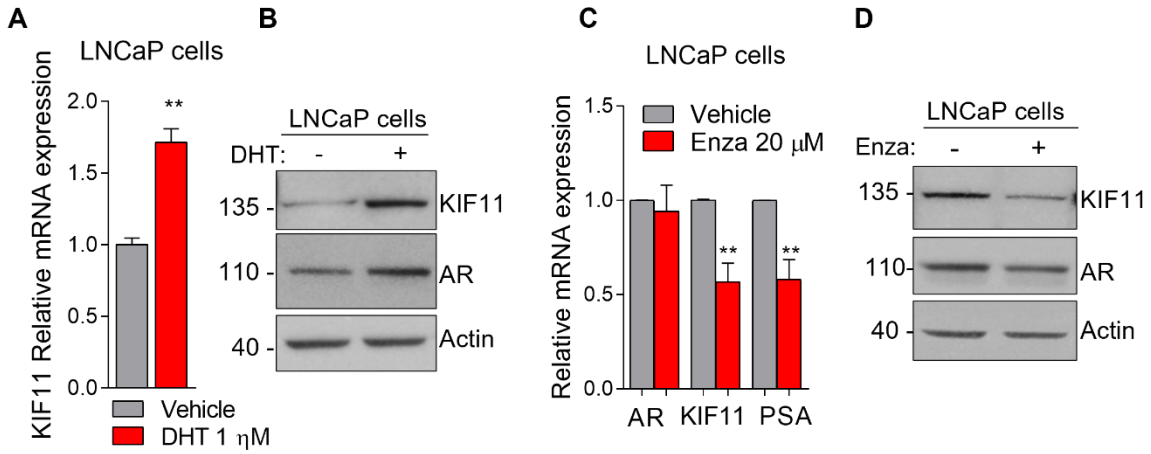

Figure 34. AR modulation regulates KIF11 protein and mRNA levels in LNCaP cells. (A) LNCaP cells were cultured in charcoal-stripped serum medium for 3 days before challenged with 1ηM DHT. After 4h of androgen stimulation, KIF11 mRNA levels were measured by RT-qPCR and **(B)** after 24h, protein levels were analysed by western blot. **(C)** KIF11 mRNA and **(D)** protein levels in LNCaP cell line after 48h treatment with 20 μM enzalutamide. Data shown are mean of three independent experiments ±SEM. **p<0.01, two-tailed Student's t-test.

To verify that the observed changes upon KIF11 expression were a consequence of AR inhibition and not due to some off-target effect of the enzalutamide, we decided to genetically knock-down AR expression using siRNAs. For that aim, we used 22Rv1 and C4-2 cell lines, two universally used CRPC models [364] that were transiently transfected with AR siRNA. After 72h of AR depletion, we observed a reduction on KIF11 protein levels in both cell lines (**Figure 35**A and B), corroborating the previous results.

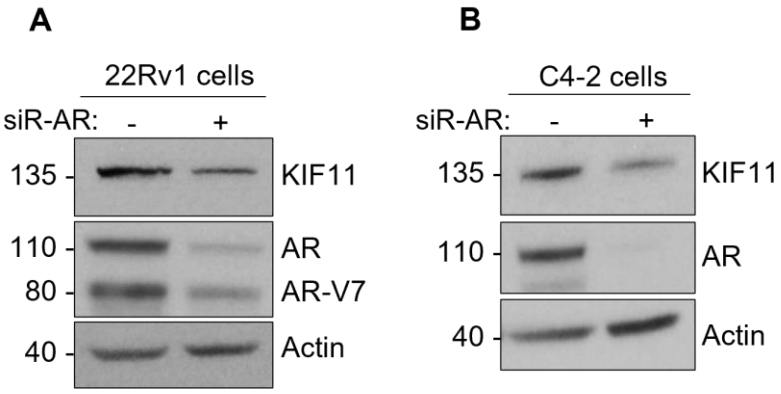

Figure 35. AR knock-down reduces KIF11 protein expression in AR-positive CRPC cell lines. Immunoblot detection of KIF11 protein level after 72h of AR siRNA knock-down in the CRPC AR-positive cell lines **(A)** 22Rv1 and **(B)** C4-2 cells.

Moreover, to confirm that the effects observed on KIF11 expression upon AR knock-down were a direct consequence of AR depletion we transfected Du145, an AR-negative PCa cell line, with this same siRNA. As expected, no changes were observed on KIF11 mRNA (**Figure 36**A) and protein (**Figure 36**B) levels 72h after transfection with the AR siRNA**.** Altogether, these findings suggest that KIF11 expression might be modulated by AR activity.

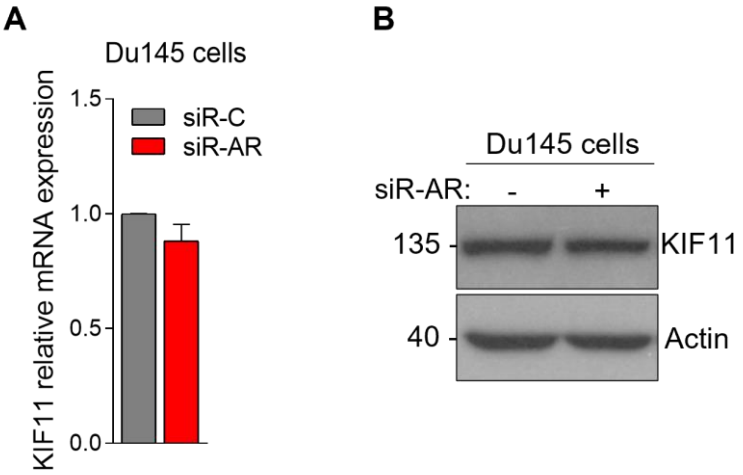

Figure 36. AR knock-down does not alter KIF11 expression in an AR-negative PCa model. (A) KIF11 mRNA and **(B)** protein levels in Du145 cell line after 72h of AR depletion with siRNA. Data shown are mean of three independent experiments ±SEM.

4.8. KIF11 modulates AR protein levels and AR transcriptional activity

Our previous results showed an association between KIF11 expression and CRPC development. Moreover, we observed a positive correlation between the expression of KIF11 and the AR, which is considered a well characterised driver of CRPC [141]. Based on that, we decided to further investigate the role of KIF11 in this disease, specifically in the context of a possible intertalk between these two proteins. To do that, we started by silencing KIF11 expression using siRNAs in the AR-positive CRPC model 22Rv1 cell line, which expresses the AR splice variant AR-V7, that lacks the ligand-binding domain and has been associated with CRPC and with AR-targeted therapy resistance [151,152]. Western blot analysis showed that after 48h of KIF11 depletion, AR protein levels decreased, as well as the levels of AR-V7 (**Figure 37**A). Furthermore, the transcriptional activity of the AR was also diminished, as shown by the reduced transcript levels of the AR canonical target genes PSA and TMPRSS2 (**Figure 37**B). As a reduction of the protein levels could be a consequence of decreased transcription, we also investigated AR and AR-V7 mRNA levels, but not significant changes were detected after KIF11 knock-down (**Figure 37**B). This made us think that some kind of post-transcriptional mechanism might be the responsible of the observed protein decline. Then, to see if the observations were a cell-specific event or a more general effect of KIF11 inhibition on the AR, we repeated the same approach in another AR-positive CRPC model, the C4-2 cell line, obtaining analogous results, reduced AR protein with no impact on mRNA levels (**Figure 37**C and D). To further confirm a possible role of KIF11 over AR activity, we decided to analyse the opposite, the effect of KIF11 overexpression on AR signalling. To do that, we induced KIF11 overexpression in the stably transfected LNCaP cell line (pINDUCER20-KIF11) during 72h. Interestingly, we observed that KIF11 overexpression produced an increase in AR transcriptional activity, reflected as an enhanced expression of the AR target genes PSA and TMPRSS2 while, once more, AR mRNA levels remained unaltered (**Figure 37**E).

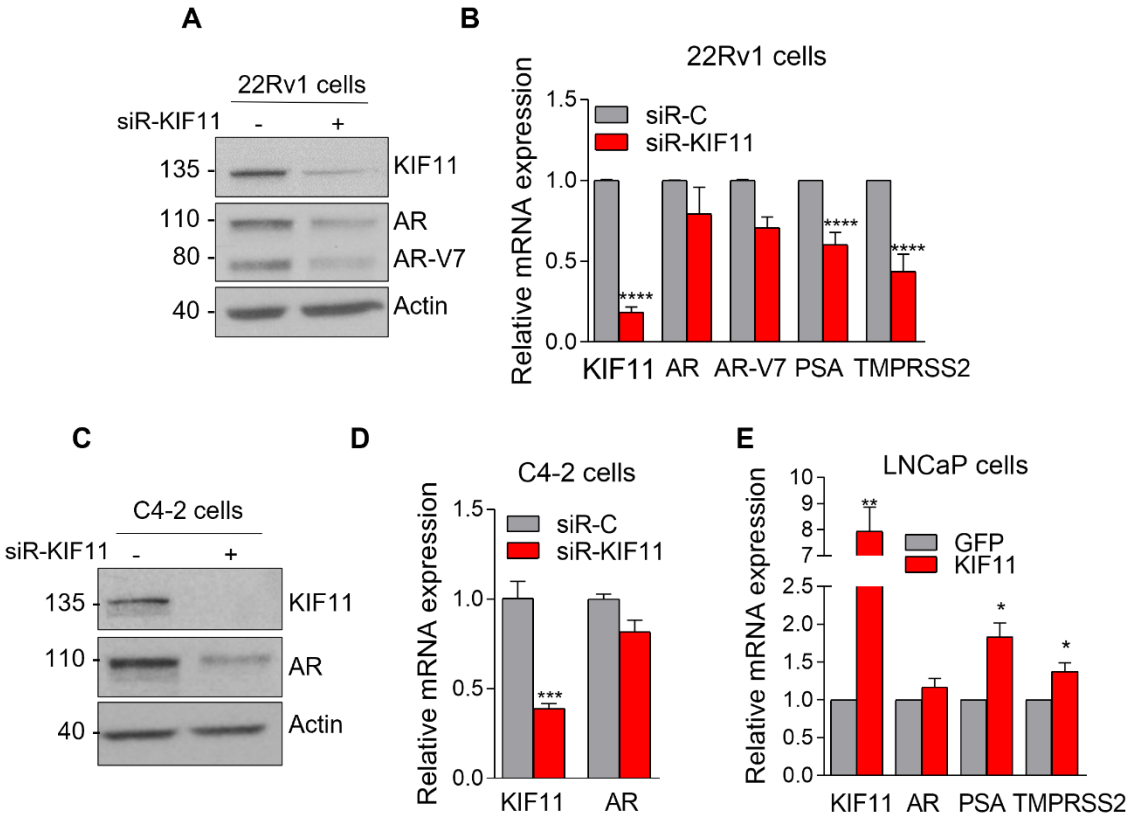

Figure 37. KIF11 modulates AR protein levels and transcriptional activity but does not alter AR mRNA levels. (A) WB detection of AR and AR-V7 protein levels after 48h of KIF11 depletion in 22Rv1 cells. **(B)** RT-qPCR analysis of the transcript levels of the AR, AR-V7 and some of the AR canonical target genes after 48h of KIF11 knockdown in 22Rv1. **(C)** Western blot and **(D)** RT-qPCR analysis of the AR protein and transcript levels after 48h of KIF11 knock down in C4-2 cell line**. (E)** KIF11 overexpression was induced with doxycycline in LNCaP stable cell line (pINDUCER20-KIF11) for 72h. After that time, expression of the indicated genes was analysed by RT-qPCR. Data are represented as mean of three independent experiments ±SEM *p<0.05, **p<0.01, ***p<0.0001, two-tailed Student's t-test.

As we were interested on proving the therapeutic potential of KIF11 pharmacological inhibition in PCa, we also studied if KIF11 inhibitor 4SC-205 was able to replicate the above noted effects of KIF11 siRNA-mediated depletion. Importantly, we observed that KIF11 inhibition by 4SC-205 was also able to reduce AR and AR-V7 protein levels in both CRPC models in a dose dependent manner (**Figure 38**).

Altogether, these results indicated that KIF11 might be modulating in some way AR signalling pathway contributing to PCa progression, and that this regulation would be taking place at a post-transcriptional level. Moreover, these findings point to a novel pathway, besides mitosis, by which KIF11 inhibition could display good therapeutic efficacy in PCa patients.

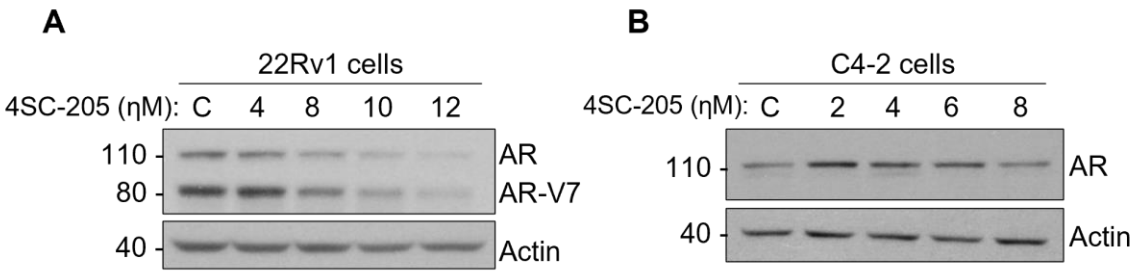

Figure 38. Pharmacological inhibition of KIF11 by 4SC-205 reduces AR and AR-V7 proteins in CRPC cell lines. (A) 22Rv1 and **(B)** C4-2 cells were treated with 4SC-205 for 48h at the indicated concentrations. AR and AR-V7 protein levels were analysed by western blot.

4.9. KIF11 depletion alters AR and AR-V7 protein stability in CRPC cells

The above results suggest that KIF11 is modulating AR protein and transcriptional activity at a post-transcriptional level. Based on that, we decided to explore if KIF11 is contributing in some way to AR protein stability. To test this possibility, we performed protein stability assays to specifically determine if AR and AR-V7 protein integrity were reduced under KIF11 depleted conditions. For that aim, we knocked-down KIF11 expression in 22Rv1 cells using siRNAs and blocked *de novo* protein biosynthesis with cycloheximide (CHX). Immunoblot analysis after 48h of KIF11 depletion and exposure to CHX for the indicated times revealed that KIF11 downregulation remarkably accelerated AR and AR-V7 protein degradation (**Figure 39**A and B). This procedure was replicated using C4-2 cell line, obtaining comparable results (**Figure 39**C and D).

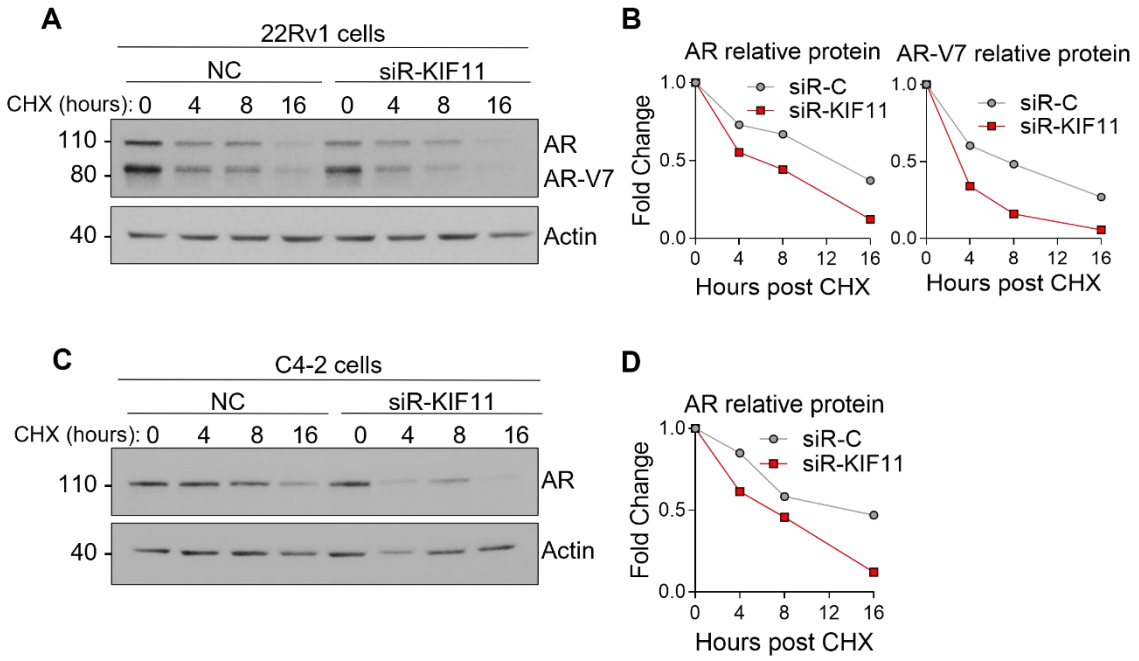

Figure 39. KIF11 depletion alters AR and AR-V7 protein stability. (A) 22Rv1 and **(C)** C4-2 cells were transfected with KIF11 siRNA for 24h and then treated with 10 µM cycloheximide (CHX) during 0, 4, 8 and 16h. AR and AR-V7 proteins levels were determined by western blot. **(B, D)** AR and AR-V7 protein quantification from (A) and (C) by densitometry analysis for each time point and cell line normalized to Actin levels, from triplicate experiments.

Then, using the same approach, we wanted to verify if KIF11 overexpression caused opposite effects on AR stability than the above-mentioned ones. We induced KIF11 expression in LNCaP stable cell line and then, cells were treated with CHX for the indicated times. As expected, immunoblot analysis showed that KIF11 overexpression reduced the rate of AR protein degradation (**Figure 40**), reinforcing the idea that KIF11 might be promoting AR protein stability.

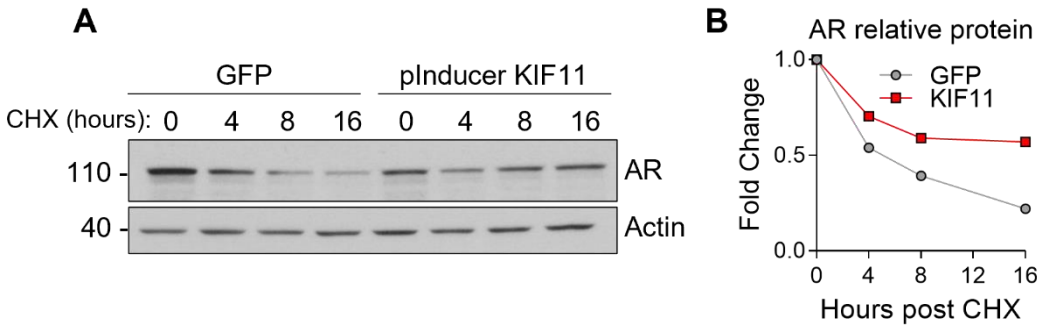

Figure 40. KIF11 overexpression in LNCaP cell line enhanced AR protein stability. (A) KIF11 expression vector was induced for 24h with doxycycline. Then, cells were treated with 10 µM of cycloheximide (CHX) for the indicated hours. AR protein bands were determined by western blot and **(B)** quantified by densitometry analysis normalized to Actin levels from triplicate experiments.

As the previously exposed results seem to point out that KIF11 regulates AR protein stability and since it is well described that the proteasome pathway mediates AR protein degradation in eukaryotic cells [385], we decided next to test the impact of proteasome inhibition on AR protein levels after KIF11 knock-down. To analyse that, 22Rv1 and C4-2 cells were transiently transfected with KIF11 siRNA and after 40h, cells were treated with the proteasome pathway–mediated protein degradation inhibitor MG132 for 4h. Immunoblot analysis showed that, as previously observed, KIF11 depletion in 22Rv1 (**Figure 41**A) and C4-2 (**Figure 41B**) cells caused a decrease on AR and AR-V7 protein levels. However, upon MG132 treatment, AR reduction induced by KIF11 depletion was rescued in both cell lines (**Figure 41**A and B).

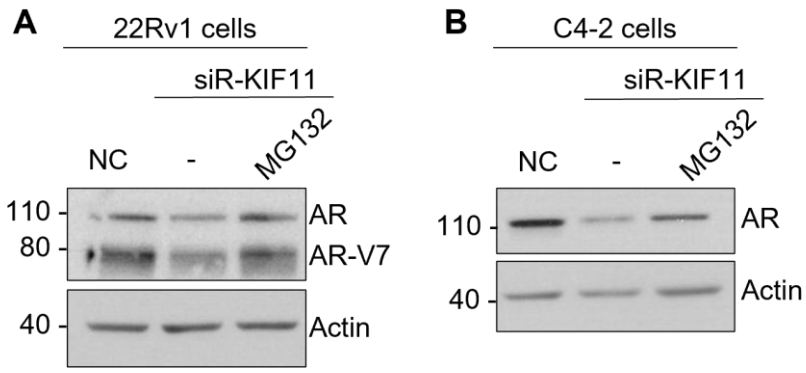

Figure 41. Proteasome pathway inhibition rescued KIF11 depletion-induced degradation of the AR and AR-V7. (A) 22Rv1 and **(B)** C4-2 cells were transfected with KIF11 siRNA for 40h and treated with 20 μM of proteasome inhibitor MG132 for 4h. AR and AR-V7 protein levels were detected by immunoblot.

Altogether, these results suggest that KIF11 is able to modulate AR signalling pathway by conferring stability to AR protein and its variants. By doing so, KIF11 might be contributing to PCa progression and to the development of AR-targeted therapy resistance.

4.10. KIF11 might influence AR and AR-V7 transcriptional activity participating on their nuclear translocation

As we have observed that KIF11 depletion and inhibition reduced AR/AR-V7 protein levels but not AR transcript, we hypothesized that KIF11 regulates AR protein through a post-transcriptional mechanism. To further study this mechanism, we decided to test a possible direct interaction between both proteins, by which KIF11 could be conferring stability to AR protein. To test this possibility, we performed co-immunoprecipitation (Co-IP) assays using both, 22Rv1 and C4-2 CRPC cell lines. First, we pulled down AR from cell lysates employing an anti-AR antibody and a possible interaction with KIF11 was analysed by western blot.

The results showed that there was not an apparent endogenous co-immunoprecipitation between these proteins (**Figure 42**A). In order to confirm these results, we performed the same experiment, this time immunoprecipitating KIF11 from C4-2 cell lysates, using an anti-KIF11 antibody (**Figure 42**B). After western blot analysis of the immune complexes, we obtained similar results, strongly suggesting that KIF11 and the androgen receptor do not directly interact.

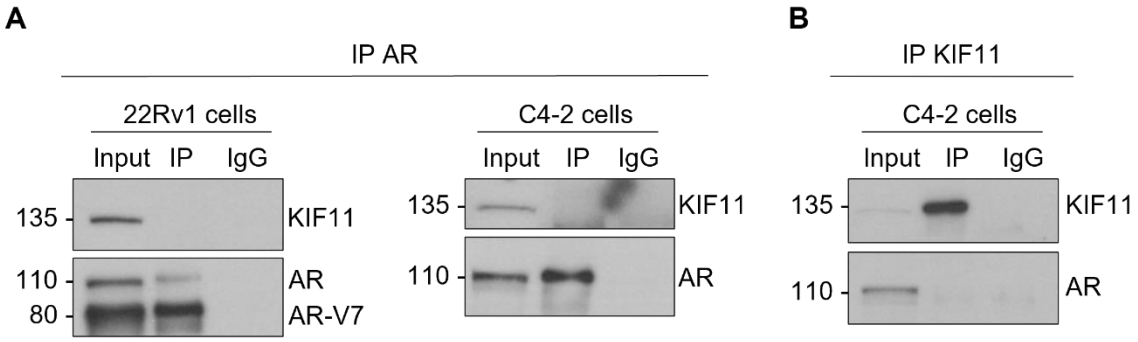

Figure 42. There is not apparent direct physical interaction between KIF11 and the AR. (A) Co-IP assays of the AR and KIF11 were performed on 22Rv1 (left) and C4-2 (right) whole-cell lysates using anti- AR antibody and blotted with the indicated antibodies. **(B)** C4-2 whole-cell lysates were immunoprecipitated with anti-KIF11 antibodies and the indicated proteins were analysed by immunoblot.

Given the previous results, we decided to explore other plausible mechanisms responsible of the attenuated transcriptional activity and enhanced degradation of the AR observed upon KIF11 inhibition/depletion. It is known that some transcriptional factors, such as TP53, the hypoxia-inducible factor 1 (HIF-1α) or the glucocorticoid receptor (GR), depend on MT cytoskeleton dynamics for their nuclear transport and to perform their function [386–388]. Moreover, some years ago, several works demonstrated that the AR also requires the MT network to be translocated into the nucleus [389–392]. As previously explained, KIF11 is a motor protein essential for the maintenance of MT structures and capable of sliding apart MT during mitosis. Moreover, throughout the last years, increasing evidence has shown that it also plays some important roles in interphase MT-related processes [327,328]. Based on all that, we thought that KIF11 could be implicated in the translocation of the AR to the nucleus through its ability to crosslink and slide apart MT, and that the impairment of this trafficking could ultimately affect AR stability and provoke its degradation. To test this possibility, we decided to study AR and AR-V7 cellular localization through nuclear/cytoplasmatic subcellular fractionation analysis in 22RV1 cells after KIF11 inhibition by 4SC-205. To enhance AR nuclear translocation and better determine the ability of KIF11 inhibition to interfere with it, cells were cultured in androgen depleted medium and stimulated with 1 ηM DHT 2h before harvested. **Figure 43** shows that, as expected AR-V7 is constitutively

located in the nucleus, independently of the presence or absence of androgens. Remarkably, upon KIF11 inhibition with 4SC-205, AR-V7 nuclear localization is drastically reduced. Predictably, androgen stimulation induced full-length AR translocation to the nucleus, which again was impaired by KIF11 inhibition. Together, these results suggest that KIF11 inhibition prevents AR and AR-V7 nuclear translocation, which in turn could eventually lead to its degradation.

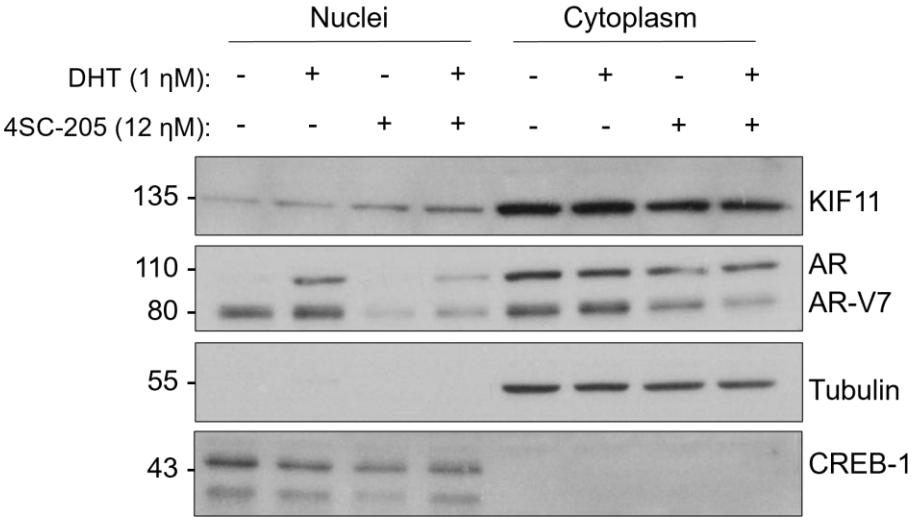

Figure 43. KIF11 inhibition with 4SC-205 impairs AR and AR-V7 nuclear translocation. 22Rv1 cells were cultured in androgen depleted conditions during 24h. Then, cells were treated with 4SC-205 at the indicated concentration for 48h. 2h before harvested, cells were challenged with 1 ηM DHT. Subcellular fractionation was performed, and the expression of the indicated proteins on each cellular compartment was analysed by western blot.

4.11. KIF11 and AR/AR-V7 may form a positive feed-back loop contributing to PCa progression

Based on all the previous results and under the already known fact that the AR requires the MT cytoskeleton to be translocated to the nucleus and to exert its function as a transcription factor [389–392], we hypothesize that KIF11 might play a critical role in this step, by maintaining an adequate MT trafficking. On the other hand, it has been demonstrated that the AR is responsible for the upregulation of a subset of mitotic proteins during CRPC development [192]. Moreover, we have shown a positive correlation between KIF11 and AR expression in PCa, which was further corroborated by several functional assays. All things considered, we believe that a positive feed-back loop between these two proteins is taking place during PCa progression. We propose that KIF11 participates in AR nuclear translocation, by crosslinking and sliding apart MT, used as “tracks” by the AR. On its side, the AR would be promoting KIF11 transcription, feed-forwarding all the previous steps along with enhancing

PCa cells division. The impairment of KIF11 functions, apart from its more evident effects avoiding mitosis, would be disturbing the MT trafficking in PCa cells, impairing AR nuclear translocation, eventually leading to its degradation. **Figure 44** illustrates a schematic representation of the described process.

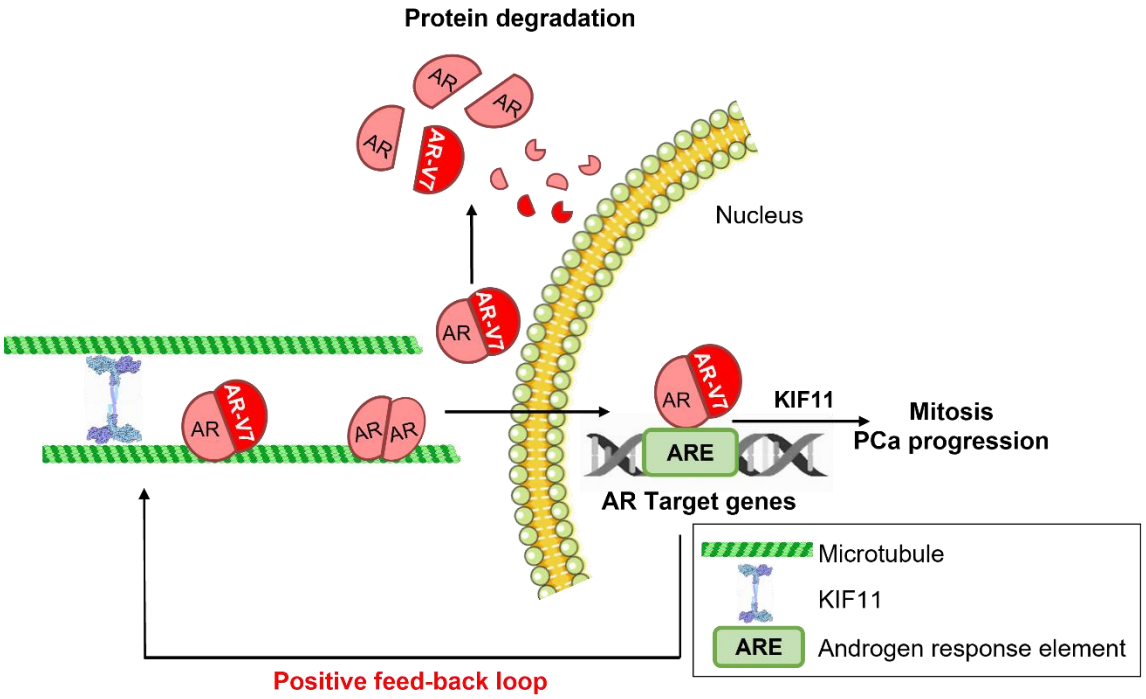

Figure 44. Schematic representation of the putative positive feed-back loop between KIF11 and AR/AR-V7. KIF11 participates in AR nuclear translocation by its ability to crosslink and slide apart antiparallel microtubules. Meanwhile, the AR promotes KIF11 expression, potentiating the previous step and enhancing cell mitosis. Altogether would be promoting PCa progression.

4.12. Combination of 4SC-205 and enzalutamide synergistically inhibits cell viability *in vitro*

Based on all the presented results, KIF11 seems to play an important role in the progression of PCa towards CRPC. We have demonstrated that KIF11 inhibition displays strong antitumoral activity both, *in vitro* and *in vivo* CRPC models. Moreover, apart from its essential function during mitosis, it seems to be implicated in the translocation of the AR and AR-V7 to the nucleus. Based on all the evidence, we have proposed a positive feed-back loop between KIF11 and AR/AR-V7, which provides mechanistic insights for the development of a combination strategy based on the dual inhibition of the AR and KIF11. Specifically, we propose that a combination of 4SC-205 with an AR-targeted therapy, such as enzalutamide, which also inhibits AR nuclear accumulation and transcriptional activity [99], could yield enhanced therapeutic efficacy, compared to any of the treatments alone.

To test that possibility, castration resistant cell models, 22Rv1 and C4-2 were treated with 4SC-205 and enzalutamide alone or in combination, using five different concentrations of each drug to identify synergy. **Figure 45**A and D shows a matrix with each paired combination that was tested and the corresponding fraction of affected cells. The inhibition of each pathway individually resulted in a moderate loss of proliferation, which was significantly enhanced upon the combination (**Figure 45**B and E). The combination indexes (CI) calculated by the Chou-Talalay method resulted in strong synergism (CI<1) for many of the pairwise combinations tested in both cell models (**Figure 45**C and F), indicating that this combination therapy could be effective for the treatment of CRPC patients.

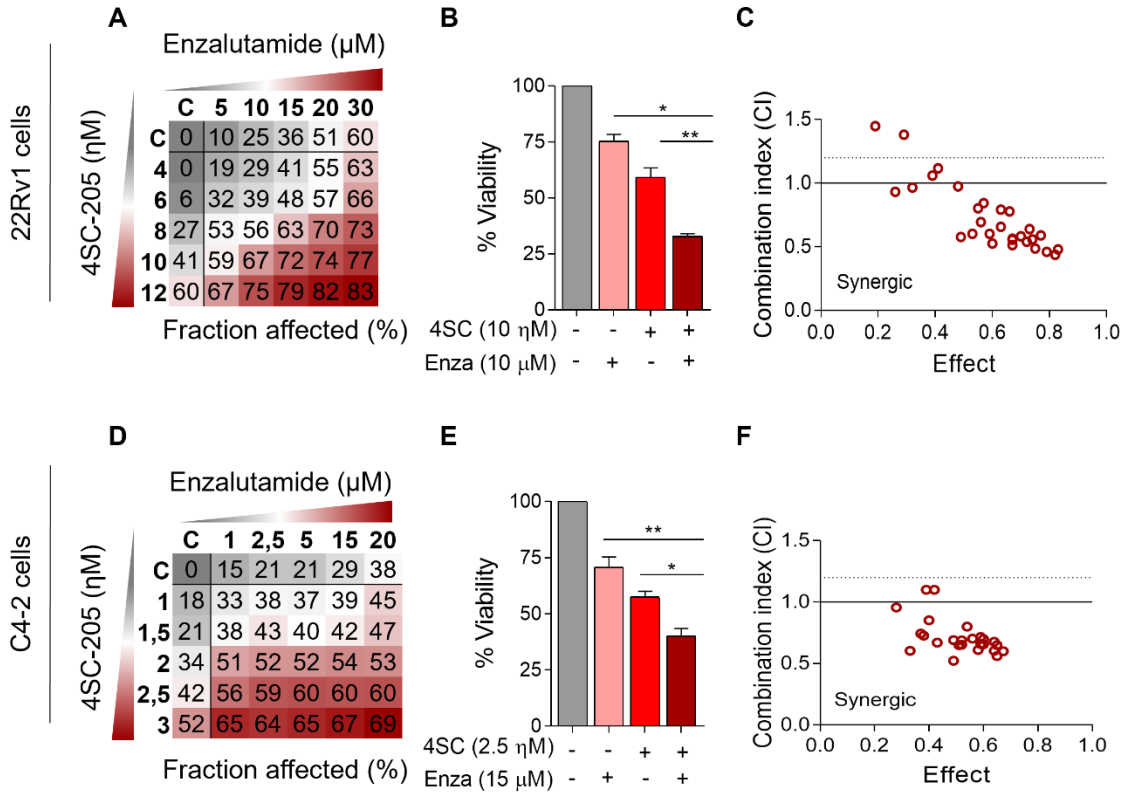

Figure 45. Combination of 4SC-205 and enzalutamide synergistically inhibits cell viability of CRCP models *in vitro*. (A, D) Cell viability data presented as a grid displaying the percentage of **(A)** 22Rv1 and **(D)** C4-2 affected cells for each pairwise combination of drug doses. **(B, E)** Strongest enhancement in **(B)** 22Rv1 and **(E)** C4-2 cell viability inhibition. **(C, F)** Combination indexes (CI) for each combination calculated following Chou-Talalay method at non-constant ratio for both cell lines. Data are represented as mean of (at least) three independent experiments ±SEM. *p<0.001, **p<0.001, two-tailed Student's t-test.

4.13. 4SC-205 and enzalutamide have synergistic effects suppressing CRPC growth *in vivo*

To validate the efficacy of the combination treatment in vivo, we used the NPp53 (Nkx3.1 $Cre^{ERT2/+}$; $Pten^{flox/flox}$; $Tp53^{flox/flox}$) genetically engineered mouse (GEM) model, which is characterized by the spontaneous development of prostate adenocarcinoma and CRPC. Co-mutations on TP53 and PTEN are rare in primary tumors but highly prevalent in CRPC [393]. Furthermore, this model has been shown to display strong molecular and phenotypic similarities with CRPC in humans and resistance to some antiandrogen therapies [373]. For these reasons, we believed that this model could be very useful to study the potential therapeutic effect of KIF11 and AR dual inhibition in CRPC. Synergistic effects of the drugs in this model were validated first *in vitro*, following the abovementioned strategy, obtaining comparable results than with the other two CRPC cell lines (**Figure 46**).

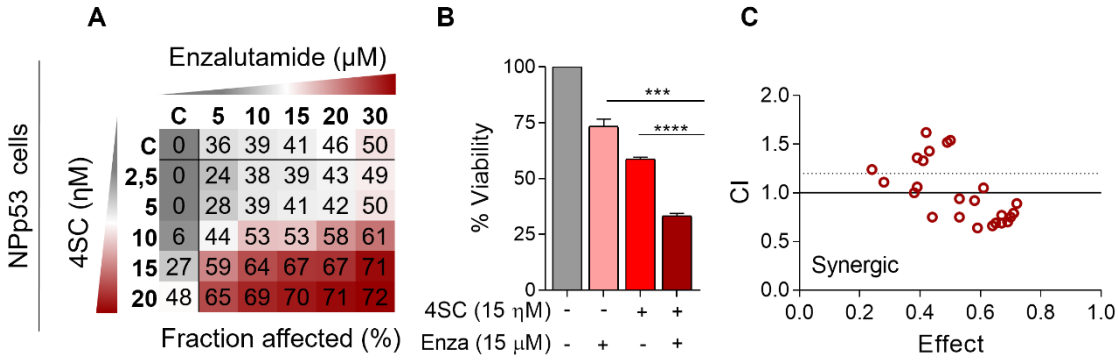

Figure 46. Combination of 4SC-205 and enzalutamide significantly enhanced antitumor activity in the NPp53 transgenic CRPC model *in vitro*. (A) Cell viability data presented as a grid displaying the percentage of NPp53 affected cells for each pairwise combination of drug doses. **(B)** Strongest enhancement in NPp53 cell viability inhibition. **(C)** Combination index (CI) for each combination calculated following Chou-Talalay method at non-constant ratio. Data shown are mean of three independent experiments ±SEM ***p<0.001, ****p<0.0001, two-tailed Student's t-test.

After that, from tumor cells derived from this model, we generated subcutaneous xenografts in immunocompromised nude mice. One week prior to cell injection, mice were castrated by bilateral orchiectomy in order to deplete androgen levels. When tumors reached 150 mm^3 on average, animals were randomly distributed in 4 experimental groups (n=7/group) and treated as follow: vehicle (1% carboxymethylcellulose, p.o), enzalutamide (10 mg/kg, p.o), 4SC-205 (20 mg/kg, p.o) or enzalutamide (10 mg/kg, p.o) + 4SC-205 (20 mg/kg, p.o) following the treatment schedule shown in **Figure 47**A during 4 weeks.

Tumors from mice treated with enzalutamide alone showed no differences in tumor volume compared with control group (enzalutamide *vs*. control; 1252.9 ± 358.9 *vs.* 1136.4 ± 216.5 mm^3). However, 4SC-205 alone was able to significantly reduced tumor growth (775.5 ± 259.5 mm^3), which was further enhanced upon the combination treatment (381.1 ± 161.1 mm^3) (**Figure 47**B). The weight of excised tumors at the end of the experiment confirmed the previous results (**Figure 47**C and D). Mouse body weight was monitored throughout the experiment with no important differences detected between groups (**Figure 47**E). Western blot analysis of representative tumors from each group showed an evident increment in the marker of mitotic arrest phospho-H3 in 4SC-205 treated tumors compared with vehicle and enzalutamide groups. Furthermore, this increase was even more pronounced upon the combination treatment, thereby indicating an enhanced antitumor activity (**Figure 47**F and G). KIF11 expression was also verified in these same tumors (**Figure 47**F).

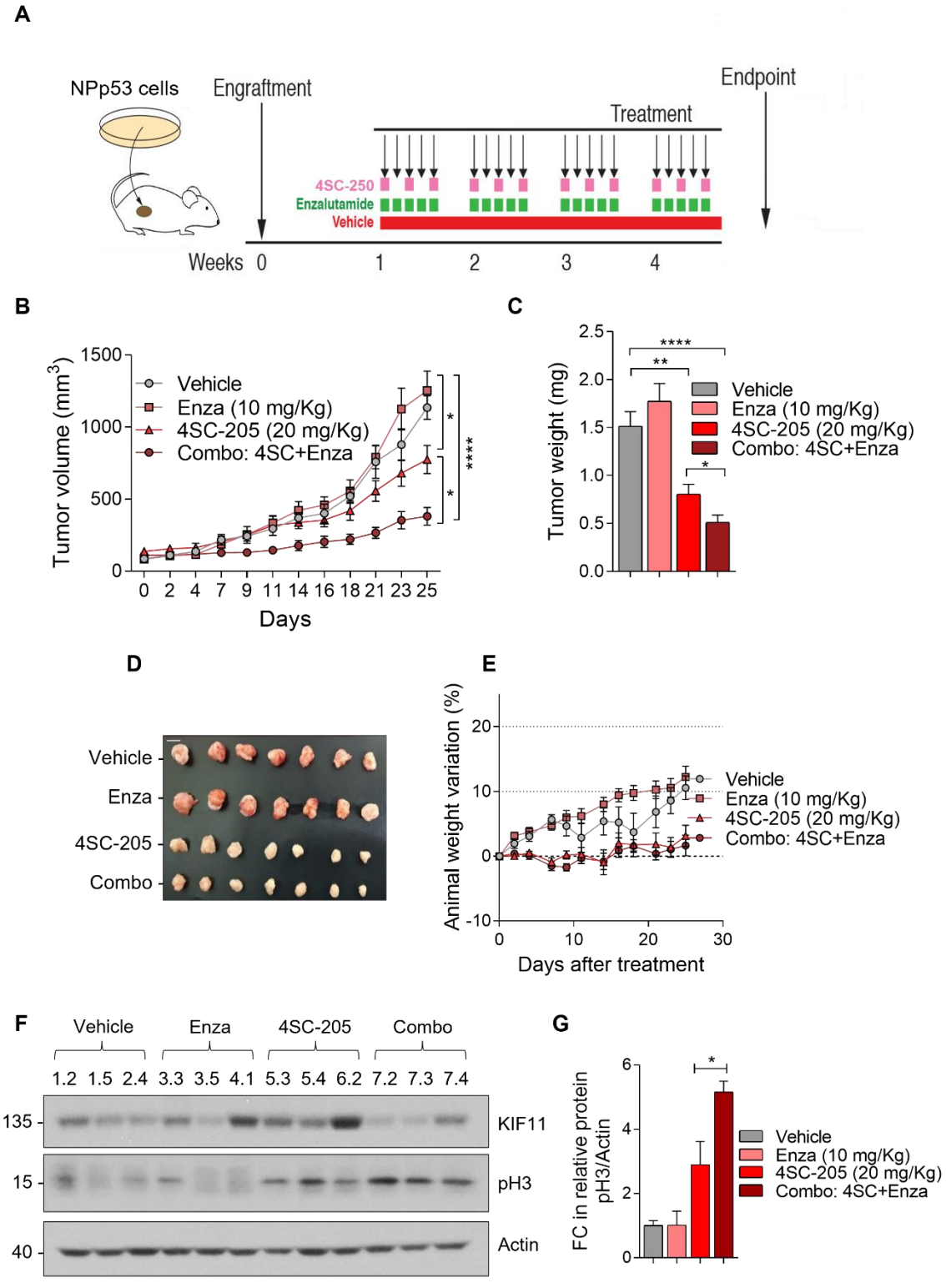

Figure 47. 4SC-205 and enzalutamide have synergistic effects suppressing CRPC growth *in vivo*. (A) Scheme of the experimental design: after establishment of tumors, mice were treated with vehicle, enzalutamide (10 mg/kg), 4SC-205 (20 mg/kg) or the combination of both. Tumors were extracted after 4 weeks of treatment. **(B)** Tumor growth curves of the transgenic murine model PTEN/P53 (-/-) in response to treatment with vehicle (n=7), enzalutamide (n=7, 10 mg/ Kg), 4SC-205 (n=7, 20 mg/Kg) or the combination (n=7) of both for 4 weeks. **(C)** Average weight of resected tumors at the end of the experiment for each treatment group. **(D)** Representative images of the

tumors at endpoint. Sacale bar: 1cm **(E)** Mouse weight variation during four weeks of oral administration of each treatment. **(F)** pH3 as a marker of mitotic arrest was analysed by immunoblot in excised tumors. **(G)** pH3 protein levels quantification from (F) by densitometry analysis. Data shown are mean ±SEM. *p<0.05, **p<0.01, ***p<0.001, two-tailed Student's t-test.

In order to verify if the effects observed on AR expression upon KIF11 inhibition *in vitro* were reproduced *in vivo*, we analysed AR protein and mRNA levels by IHC and RTq-PCR, respectively on representative tumors from each group. The results showed a reduction in AR protein upon 4SC-205 and the combination treatment. No effect on AR protein levels were observed in enzalutamide or vehicle treated tumors (**Figure 48**A). AR mRNA levels did not change significantly upon any of the treatment groups (**Figure 48**B), in accordance with the results of the *in vitro* assays.

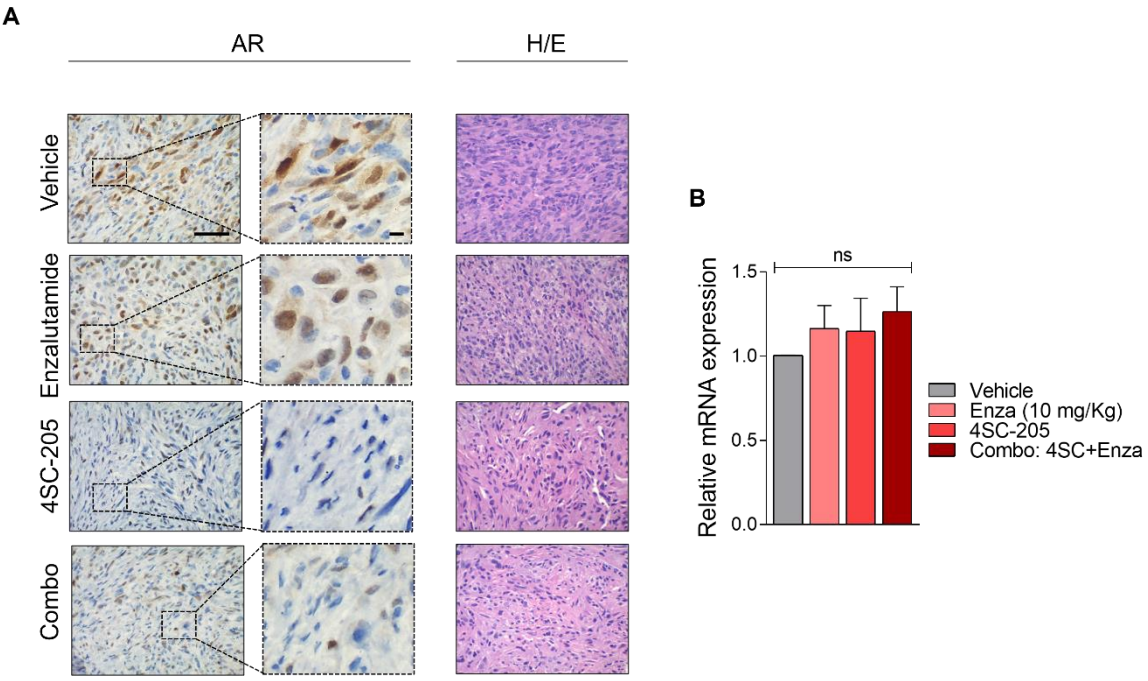

Figure 48. KIF11 inhibition with 4SC-205 reduced AR protein expression but not AR transcript levels *in vivo*. (A) Representative IHC staining images of AR and H/E on tumor slides from each group. Scale bar, 50 µm. Inset scale bar 10 µm. **(B)** RT-qPCR analysis of AR mRNA expression in representative tumors from each group.

Altogether, these data suggest that combined inhibition of KIF11 and the AR synergistically inhibits PCa growth both, *in vitro* and *in vivo*, and gives a good rationale for a combination therapy using 4SC-205 and enzalutamide for the treatment of CRPC patients.

5. DISCUSSION

5.1. Challenges of PCa clinical management

Prostate cancer is the second most commonly diagnosed malignancy and the fifth cause of cancer-related deaths among men worldwide [19]. Important advances in screening and diagnosis techniques have led to improve early detection, which have been critical to increase PCa survival. Patients diagnosed with tumors confined in the prostate displayed 5-years survival rates of around 100%, as the disease at these stages can be definitively treated. However, 20 to 30% of patients that are diagnosed with localized disease will experience recurrence [394] and around 5% of men will be diagnosed with already advanced disease[27], for whom the available treatments are not curative and their 5-year survival rate drops below 30%. Given the important role that androgens and the AR play in the progression of PCa, for several decades ADT has been the "gold standard" treatment for advanced PCa [137,138]. Despite its initial effects stabilizing or causing disease regression with PSA and tumor size reduction, almost all patients progress to CRPC, which is the major clinical challenge in the management of PCa and remains incurable [395]. It has been proven that the AR signalling pathway is still important for PCa progression at these stages, despite depletion of serum androgen levels [384]. So much so that first-generation antiandrogens such as bicalutamide, and novel improved derivatives like enzalutamide, have been the standard of care for CRPC patients for many years, showing significant survival benefit and improved clinical outcomes [396,397]. However, despite initial response, most patients eventually develop resistance, and their median survival is lower than 2 years [139]. Therefore, understanding the molecular bases underlying the development of CRPC and therapy resistance is an urgent need in order to identify new therapeutic targets to treat this lethal stage of the disease.

During the last years, advances in cancer genomics and proteomics have provided novel insights about the molecular basis of PCa that are helping to improve and facilitate therapy decision-making. A clear example are DDR gene defects, such as those affecting BRCA1/2, ATM, and CHECK2 among others [58]. The high frequency of these alterations detected on advance PCa patients have led to the approval of the recommendation of germline testing for DDR genes in all patients with advanced or high-risk localized disease [115]. Such testing would give useful information for treatment decision-making. For instance, patients harboring mutations in homologous recombination repair (HRR) genes have been shown to benefit from the treatment with PARP inhibitors, such as Olaparib, as they cause synthetic lethality in cancer cells with these alterations [112–114]. Another example is the improved performance of immune checkpoint inhibitors, such as anti-PD-1 antibody pembrolizumab, in a subset of patients with MSI due to alterations on the MMR system [119]. Likewise, a subset

of patients harboring mutations in CDK12, a gene related with genomic instability, also performed better response to immunotherapy [398]. This increased sensitivity to immunotherapy in these patients was associated to a higher gene fusion-induced neoantigens due to elevated genomic instability [120]. However, despite clear survival benefit of these treatments in these subsets of patients, they represent only a small proportion of the total PCa cases. A better understanding of the molecular biology of the disease is indeed needed in order to generate more targeted and gene-specific therapies from which a higher proportion of patients can benefit.

Targeting mitosis is one of the main therapeutic approaches used for the treatment of CRPC after the emergence of antiandrogen therapy resistance. Specifically, taxanes are the current agents used for patients with ARTA-refractory CRPC, as they were the first systemic therapy to demonstrate survival benefit on these patients [399]. However, despite initial clinical activity, patients eventually develop acquired resistance and an important fraction display no initial response [400]. These resistances, along with the severe side effects of taxanes, make evident the need for the development of novel mitotic inhibitors with improved specificity for tumor cells. In line with that, during last years several works have demonstrated that during PCa progression towards CRPC, a transcriptomic reprogramming characterised by an overexpression of M-phase cell cycle genes is taken place [192], further supporting that mitosis and mitotic proteins are interesting targets for the development of drugs to treat this disease.

To further study the implication of mitotic proteins in the acquisition of androgen independence, in a previous study from our group, we validated by quantitative proteomics the involvement of these proteins in the development of hormone independence. It was, to the best of our knowledge, the first time that this phenomenon was analysed at protein level (manuscript in preparation). In accordance to the previously mentioned transcriptomic studies, we found that M-phase cell cycle proteins were upregulated in the androgen independent models. Moreover, among the most upregulated mitotic proteins, we found several mitotic kinesins. As mentioned in previous sections, kinesins are microtubule-based motor proteins that play important functions in intracellular transport and cell division and many of them have been considered potential therapeutic targets in several tumors [243]. Moreover, some have already been related with CRPC and endocrine therapy resistance [262,277,286]. In particular, we focused our attention on KIF11, which has been previously associated with PCa progression and aggressiveness [345,346,401] however, data regarding a direct implication of the motor protein with CRPC development have not been documented to date.

Overall, we considered that the expression of this mitotic kinesin might be implicated in the reprogramming process that occurs during PCa progression toward CRPC, making it an attractive therapeutic target for the treatment of patients with this disease.

5.2. KIF11 as a marker of disease aggressiveness, CRCP and therapy response

KIF11 is a protein essential for the establishment of the bipolar spindle and mitotic progression [321]. KIF11 homozygous depletion in mouse models was shown to be lethal during embryonic development [402]. Also, several studies using next-generation sequencing techniques have pointed out that KIF11 mutations are linked with central nervous system congenital human syndromes [403–405]. However, KIF11 is non-essential in terminally differentiated adult tissues and its expression is low, especially when compared with highly proliferating and malignant cells [361]. All these indicate that the protein is crucial specifically during early phases of development and in rapidly dividing tissues, facts that are in line with its critical role during mitosis. On the other hand, overexpression of KIF11 in transgenic mouse models was demonstrated to produce chromosome miss-segregation and genetic instability that eventually led to higher rates of tumor formation [342]. Moreover, KIF11 upregulation have been linked with the development and aggressiveness of many types of cancer and has also been correlated with worse clinico-pathological characteristic and poor clinical outcome in multiple neoplasms (**Table 6**). In the specific case of PCa, KIF11 expression has been associated with poor prognosis, elevated PSA levels, higher GS and TNM stages, and metastatic disease [345,346,401]. Moreover, the work from Wissing and colleages [345] is, far as we are aware, the only evidence that points towards a possible link between KIF11 and CRPC. They suggested that hormone-naïve patients whose tumors displayed higher KIF11 expression performed shorter overall survival due to a more rapidly progression towards mCRPC. In line with that, our results demonstrate that castration resistant PCa cell models displayed higher KIF11 mRNA and protein levels compared with the castration sensitive one. Moreover, results from an extensive *in silico* analysis using multiple PCa public databases revealed that KIF11 expression was significantly higher in metastatic CRPC compared with localised PCa, benign conditions and even when compared with castration sensitive metastatic disease. Also, patients with high KIF11 expression displayed worse disease and progression free survival, suggesting that high KIF11 levels are associated with more aggressive forms of the disease. High expression of KIF11 was also found to be associated with biochemical recurrence and higher Gleason scores.

Additionally, we performed a clinical validation using representative PCa tissue samples from different stages of the disease. KIF11 mRNA levels were analysed in primary tumor samples from patients that developed or not metastatic disease. Analysis of these samples revealed that patients who progressed to metastatic disease displayed higher KIF11 expression in their primary tumors compared to those who did not. Also, protein analysis performed by IHC revealed that samples from CRPC patients presented significantly higher levels of KIF11 compared with treatment-naïve patients and benign conditions. Moreover, IHC analysis of biopsy samples from patients diagnosed with advanced disease revealed that those who became resistant to ADT in less than one year exhibited higher KIF11 levels at initial diagnosis than those who still responded after two years of treatment. Altogether, these findings suggest that KIF11 expression might be a useful marker of PCa aggressiveness, CRPC and metastatic disease development and an indicator of therapy response. However, clinical validation with a bigger cohort of patients is needed to confirm the potential of KIF11 as a biomarker.

5.3. KIF11 expression and its implication in the development of CRPC and therapy resistance

All the aforementioned seems to point towards a correlation between high KIF11 expression, CRPC and therapy resistance. However, no evidence has been described supporting a direct functional role of the protein in these events.

Plenty of works have performed loss-of-function assays demonstrating a role of KIF11 in cell proliferation, migration, invasion and in the acquisition of other malignant characteristics [368,406–408]. However, there are few data describing the consequences of KIF11 overexpression [342,357,409]. For instance, Liu and colleagues demonstrated that KIF11 overexpression in glioma cells promoted soft-agar colony formation, spheroid anchorage-independent growth ability and chemoresistance [357]. Yet, no previous work has studied that matter in the context of PCa and castration resistance acquisition.

In this work, to further study the implication of KIF11 in the gain of androgen independent characteristics and therefore, the development of CRPC, we generated a stable cell line able to overexpress KIF11 protein under doxycycline induction. For that aim, we used LNCaP cell line, which is an androgen dependent PCa model with low endogenous levels of the protein. We observed that KIF11 overexpression conferred these cells a significantly higher ability to grow in androgen depleted conditions compared with control, becoming the first functional evidence that suggests a role of the protein in the acquisition of castration

resistant features. This finding turns KIF11 into a promising target to treat this lethal disease as its inhibition could, at least in part, revert the aggressive castration resistant phenotype.

We have showed several evidence indicating that KIF11 might be implicated in CRPC and ADT resistance, however, there are no data supporting a specific function of the protein in the development of resistance to other hormonal therapies, such as enzalutamide. This is of special importance because, after PCa progression on ADT and the clinical diagnosis of CRPC, second-generation antiandrogens, such as enzalutamide are nowadays one of the standard of care treatments for these patients. However, as mentioned before, despite favourable initial response, patients eventually develop resistances and moreover, not all patients benefit from these agents [410]. Some of the molecular mechanisms described as responsible of the development of resistance to second-generation antiandrogens seem to be quite overlapping with those described in patients that become refractory to ADT [411]. These cross-resistances can be explained as both therapies, although in a different way, target the AR signalling pathway. Based on that and on all the previous evidence pointing out a role of KIF11 in the development of ADT resistance, we wanted to investigate a possible link between KIF11 expression and resistance to second-generation antiandrogens. After the analysis of KIF11 expression using databases containing transcriptional information from enzalutamide-resistant PCa cell lines and xenograft models, we observed increased expression of the protein in the resistant models compared to their parental counterparts. Moreover, after prolonged treatment of the CRPC cell line C4-2 with enzalutamide, KIF11 mRNA and protein levels were increased in a time-dependent manner. Although not at all a definitive prove, this is the first evidence indicating that continuous treatment with enzalutamide increases KIF11 expression, fact that could contribute to the development of resistance. Moreover, some recent works have described a connection between the overexpression of some other mitotic kinesins and the emergence of endocrine-therapy resistance [262,286,412].

5.4. Targeting KIF11 as an anticancer therapy

5.4.1. Performance of KIF11 inhibitors in clinical trials

KIF11 inhibitors have shown high specificity inhibiting the enzymatic activity of the protein at nanomolar concentrations and displayed promising results in multiple preclinical models both, *in vitro* and *in vivo*, impairing tumor growth [380,413,414]. Most of them also showed encouraging results in early clinical development, as they were well tolerated being among the most common side effects anemia and neutropenia. However, in subsequent phases of

clinical trials many of them showed limited efficacy and their clinical development was abandoned [361].

Ispinesib (or SB-715992) was the first KIF11 inhibitor to enter clinical trials. In general, the agent was well tolerated, being neutropenia the major side effect. Other haematological toxicities, such as anemia, leukopenia or lymphopenia were also common. However, administered as monotherapy, ispinesib failed to show significant clinical response. In general, best outcome achieved was stable disease (SD) in several malignancies such as hepatocellular cancer (46% of patients) [415], head and neck (25%) [416], renal cell carcinoma (31%) [417]or melanoma (35%) [418]. Median time to progression was short in all cases and overall survival was not improved compared to standard therapies. Due to lack of activity, trials were stopped. Somewhat better responses were observed in a clinical trial that enrolled breast cancer patients. 20% of those showed partial response (PR), 60% SD during more than 40 days, and 27% achieved SD for more than 90 days [419].

In line with the observed for ispinesib were the results of a phase I trial with SB-743921. The study included patients with advanced solid tumors. 6 out of 41 patients (15%) performed durable stable disease. Only one patient with cholangiocarcinoma achieved PR during almost 12 months [420]. The major dose-limiting toxicity (DST) related with SB-743921 treatment was neutropenia, as it is as well for most KIF11 inhibitors. Based on that, a phase I/II trial was carried out to assess if the addition of granulocyte colony-stimulating factor (G-CSF), a cytokine that triggers the proliferation and differentiation of the neutrophil granulocyte lineage, was able to increase the maximum tolerated dose (MTD) of the inhibitor by mitigating its DLTs. Interestingly, the MTD in the cohort without G-CSF support was of 6 mg/m^2 while for the cohort supplemented with the cytokine was of 9 mg/m^2, which represents a 50% increase. Efficacy results for this study revealed that 4 patients out of a total of 56 (7%) achieved PR and 19 (34%) performed SD [421]. Altogether, this study demonstrated that the addition of G-CSF to the treatment with SB-743921, and potentially to other KIF11 inhibitors, could mitigate the associated DLTs, increasing the MTD, and eventually improving the efficacy of the drug.

In a phase I trial, 22 patients with taxanes-resistant advanced solid tumors were treated with KIF11 inhibitor MK-0131 [422]. No objective tumor response was detected. Only 4 patients out of the total (18%) displayed prolonged SD. The major DLT was neutropenia [423]. Similarly, of a total of 48 patients treated with ARQ-621, only 6 (12.5%) achieved SD for over 4 months. In this case, neutropenia was not a DLT [424]. A phase I trial with AZD4877 [425] in lymphoma and refractory solid tumor patients no objective response was detected. Only 4 patients of a total of 22 (18%) performed prolonged SD [426]. A multicentre phase I/II

study carried out with the same agent in patients with refractory acute myeloid leukaemia showed no signs of remission or objective response. The trial was terminated due to lack of efficacy [427]. In a phase II trial in patients with recurrent urothelial tumors treated with AZD4877, only 1 patient of 39 (2.5%) displayed PR and 7 patients (18%) showed SD for over 8 months. The most common treatment-associated adverse event was neutropenia [428]. LY2523355 (or litronesib) [429] also failed to show encouraging results. In a phase one trial in patients with advanced and metastatic solid tumors refractory to standard therapies, best response was SD in 2 of 7 evaluated patients (28.5%), the longest lasting 52 days [430]. Similarly, in another phase I study enrolling 86 patients with advanced refractory solid tumors, only two of them (2.3%) accomplished PR, and 17 (20%) performed SD for more than 6 cycles. Again, neutropenia was the main DLT, which was mitigated with G-CSF allowing the administration of higher doses [431].

ALN-VSP is a formulation composed by two siRNAs targeting the vascular endothelial growth factor (VEGF) and KIF11, encapsulated into lipid nanoparticles (LNP). It was tested in a phase I clinical trial that enrolled 37 patients with advanced metastatic tumors. The drug was well tolerated and was demonstrated to reach and perform iRNA-mediated mRNA cleavage in hepatic and extrahepatic lesions. Remarkably, complete response was observed in one patient with endometrial cancer with multiple hepatic and lymph node metastasis who had progressed on chemotherapy and on another experimental treatment. Moreover, three additional patients achieved SD, two of whom, with metastatic renal cell carcinoma, displayed SD for 8-12 months. One patient with pancreatic cancer had disease control for over 18 months [432]. These results make evident that higher antitumoral activity might be achieved by combining agents targeting different pathways and highlight the importance of exploring novel rational drug combination strategies. Unfortunately, despite promising results, no further clinical trials have been performed with this agent.

ARRY-520 or filanesib, a potent KIF11 inhibitor, has been tested in multiple preclinical studies [414,433] and has shown to be especially effective in hematological malignancies, achieving 100% of complete response in some models [413]. The good results obtained in preclinical studies prompted to the analysis of its efficacy as monotherapy in the clinical setting. It was firstly tested together with low doses of corticosteroids in refractory acute myeloid leukemia patients. Filanesib showed good tolerability but only one of the 34 evaluable patients achieved PR (3%) and 10 (28%) SD [434]. The agent was also tested in patients with advanced heavily pre-treated solid tumors. Again, filanesib showed a good safety profile but the best response observed was SD in 7 patients of a total of 39 (18%) [435]. More encouraging results were obtained in a phase I/II trial performed with filanesib as

monotherapy in multiple myeloma (MM) patients who had progressed on at least two prior treatments. The observed overall response rate (ORR) (≥ partial response) was of 16%. The responses were durable with a mean overall survival of 19 months [436].

The promising results observed for filanesib as single agent in MM patients encouraged the development of combination studies that could potentially improve efficacy. Specifically, research has been focused on combinations with proteasome inhibitors (PI) and immunomodulatory agents (IMiDs) that during the last years have shown to improve survival of recurrent/refractory MM (RRMM) patients [437,438]. Moreover, results from preclinical studies seem to support them as rational combinations and prompted their clinical evaluation [439]. The combination of filanesib with the PI inhibitor bortezomib and low doses of corticosteroids (dexamethasone) was evaluated on a phase I trial in RRMM patients. The combination was well tolerated with manageable haematological toxicities (using prophylactic G-CSF). Efficacy results were promising. Of a total of 19 evaluable patients treated with an specific schedule, one achieved complete response (5%), 4 very good PR (VGPR) (21 %) and 3 PR (16%) [440]. Similar results were observed on a phase I study using filanesib and the second-generation PI carfilzomib in 64 RRMM patients. There was an ORR (≥ partial response) of 37% of which 29% were PR and the remaining 8% VGPR [441]. The efficacy of filanesib was also tested in combination with the IMiDs pomalidomide plus dexamethasone in a phase Ib/II trial including MM with progressive disease after several lines of treatment. Results were also encouraging. Among the 26 evaluable patients, the ORR was of 65% (≥ partial response) of which 11% (3 patients) were VGPR and 54% (14 patients) PR. The efficacy of the combination was clearly superior to that of pomalidomide plus dexamethasone alone with an ORR of 30% [442]. Moreover, in many of the abovementioned trials with filanesib it was observed that patients with elevated serum alpha 1-acid glycoprotein (AAG) had milder response to the drug than those with low levels. It was thought that the agent binds to this protein and the complex is rapidly eliminated, avoiding filanesib to exert its function, reducing its efficacy. This turns serum AAG levels into a potential biomarker of response to this agent and make evident that specific characteristic from certain patients can make a particular drug less effective. It would be highly helpful to unveil these particular traits in order to select the most appropriate therapy for each patient, moving towards more individualised treatment approaches.

Altogether, the main objective of the development of these targeted drugs was to overcome the severe side effects associated with chemotherapeutics, such as neurotoxicity caused by tubulin targeting agents. This problem seems to be overcome as in none of the trials using KIF11 inhibitors neurotoxicity was reported, not even in the cases that were considered more susceptible due to intense prior treatment with neurotoxic agents. Other

non-related haematological toxicities were rare. However, the antitumor activity of these drugs observed in clinical phases was quite disappointing. In general cell cycle/mitotic inhibitors have small therapeutic windows in the clinics, due to their toxic effects on high proliferative normal tissues [443]. In the case of KIF11 inhibitors, neutropenia was the most common DLT, and it has been an obstacle when trying to widen the therapeutic window, limiting the clinical development of these agents. The addition of bone marrow stimulating cytokines, such as G-CSF, was able to alleviate this adverse event allowing increasing the MTD. These, along with the development of new rational combinations with standard chemotherapy or other targeted therapies could help to potentiate the antitumor activity of these drugs, which could ultimately improve their efficacy, as it is being tested in clinical and preclinical studies [368,444–446].

5.4.2. Why great inhibitors make poor anticancer drugs

Initially, one could think that the low response rates performed by the abovementioned KIF11 inhibitors could be due to low specificity or bioavailability of the drugs. However, this possibility is dismissed, as in most cases an increased in mitotic arrest biomarkers (mitotic index and phosphorylation of H3) was observed upon treatment, indicating a specific drug activity [447]. Moreover, the common neutropenia observed in most treated patients is another evidence that the drugs were reaching the target, in fact it was proposed as a useful biomarker of activity of this class of inhibitors [420].

Pharmacokinetics and the proliferation rate paradox

One reason for the failure could be related with the fact that the proliferation rate of solid tumors is slower than it has been traditionally thought. The median doubling time for several types of tumors is 50-80 days [448], much longer than the observed doubling time in preclinical *in vivo* models (3-6 days) [449] and in prolific non-tumoral tissues such as the bone marrow (24h) and the gut epithelium (3 days) [443]. This means that in a tumor at a given moment only a small fraction of cells (around 1%) would be in mitosis, phenomenon known as the "mitotic rate paradox" [450]. As the expression of the target of most of the currently available mitotic inhibitors is restricted to mitosis, the specific agent should be long enough in the serum of the patient to reach a higher proportion of mitotic cells and eventually hit the target. If not so, most tumor cells would be indifferent to the effect of these drugs [361]. Supporting this possibility is the higher efficacy performed by filanesib in MM patients. Considering that highly proliferative hematopoietic cells are greatly sensitive to this class of inhibitors, it makes sense that rapidly dividing myeloma cells are more susceptible than the slower dividing cells found solid tumors. Moreover, filanesib has a longer half-life than other KIF11

inhibitors, which allows the drug to remain longer in contact with tumor cells, increasing the chance of reaching cycling cells. All these factors may be playing a key role in the higher antitumor activity performed by filanesib in MM patients.

In line with that, Arbitratio and collages [451] demonstrated that frequent administration of SNS-314, a mitotic inhibitor targeting aurora kinases, had significantly greater effect impairing tumor growth than once weekly dosing schedule in a preclinical *in vivo* model. Also supporting these observations are the results performed by the KIF11 inhibitor 4SC-205 in clinical studies. This agent has the peculiarity of being the only KIF11 inhibitor in clinical development that can be orally administered, which allows a more frequent dosing schedule [452]. In fact, results from a phase I trial demonstrated that 20 mg once daily administration was able to overcome the "proliferation rate paradox" in solid tumor, showing a clinical response on 67% of patients performing stable disease for more than 100 days [363]. Based on this evidence, it is thought that a frequent administration of these drugs could be also a critical factor to increase the chances of reaching the target and ultimately improve drug efficacy of anti-mitotic therapies, and more specifically, KIF11 inhibitors.

Narrow therapeutic window due to adverse events on non-tumoral highly proliferative tissues

Another possible reason of the clinical failure of these agents is their toxicity over prolific non-malignant tissues. The most common DLT related with KIF11 inhibitors is neutropenia. Although it could be alleviated with G-CSF, allowing to increase the MTD, other adverse events affecting highly proliferative tissues such as the hematopoietic, gastrointestinal tract, mucous membranes or the skin, arose limiting their tolerated doses and ultimately their efficacy [443].

It is nonetheless intriguing that while novel mitotic inhibitors have failed to show a clinical impact due to, at least in part, narrow therapeutic windows, classic cytotoxic chemotherapies, which performed similar adverse events on highly proliferative non-malignant tissues (bone marrow suppression, stomatitis, infertility, severe diarrhoea, etc.), have demonstrated meaningful clinical results. To explain this, it is important to consider that when some of these chemotherapeutic drugs were first tested in the early 1950s, also failed to show significant antineoplastic activity, as their highly severe adverse events limited their used [443,453,454]. One way to reduce their DLTs was to improve their formulation developing prodrugs that were preferentially bioactivated in tumoral cells compared to normal ones. One example of this are nitrogen mustards, DNA alkylating agents that although they performed promising antitumoral activity [455,456], the associated unbearable

adverse events barred their utility [457,458]. Cyclophosphamide is a sophisticated prodrug derived from nitrogen mustards, which displays increased selectivity for tumoral cells compared with normal proliferating ones. This differential selectivity is based on the downregulation of the enzyme aldehyde dehydrogenase (ALDH) in some cancer types, which leads to agent trapping and higher DNA damage [459,460]. Another example are nucleotide analogues, as the well-known 5-fluorouracil (5-FU). This class of agents are administered as nucleosides or nucleobase analogues, which are therapeutically active only when they are transformed into nucleotide analogues that impair DNA replication [443]. This crucial step is catalysed faster in cancer cells due to a higher expression of biotransforming enzymes compared to proliferative normal cells, providing higher tumor selectivity and therefore a greater therapeutic window [443,461].

Targeted antimitotic-therapies, and specifically KIF11 inhibitors, have shown high specificity for their target, indicating that not improvements in that sense are needed. An interesting way to potentiate their efficacy could be the generation of prodrug of these agents. By doing so, target specificity would be narrowed at two levels: one, by the outstanding affinity of these drugs for their mitotic targets, only present in cycling cells; two, by the activation of the drug only in those cells with specific metabolic characteristics, avoiding their activation in prolific normal cells, and ultimately reducing the undesirable side effects. However, although very interesting, much work has to be done in this regard, as this approach requires a great knowledge of the intrinsic characteristics of each tumor type.

KIF11 point mutations

Most patients that have been enrolled in clinical trials with KIF11 inhibitors are patients with recurrent and highly aggressive tumors that have been heavily pre-treated with a wide range of chemotherapeutic agents. Based on that, a proposed possibility of the low antitumor activity performed in the clinic by KIF11 inhibitors is the pre-existence of mutations affecting the tumor sensitivity for these compounds [462]. For instance, mutations affecting the L5 loop pocket of the protein, where most KIF11 inhibitors bind, could lead to reduced affinity between the protein and the compound, conferring resistance to inhibition [463]. Some preclinical studies have demonstrated that certain mutations, particularly amino acid substitutions and deletions affecting the drug binding pocket, can confer resistance to some of these agents such as filanesib, STLC, monastrol and ispinesib [464–467]. However, no data from clinical trials with this class of agents have described or confirmed any relevant mutation that could be responsible of the poor antitumor activity. Moreover, most of the preclinical studies describing mutations involved in this resistance are based on genome editing, intense mutagenesis and/or clonal selection of cells after prolonged and intensive

exposure to the inhibitors [463,467]. Altogether, mutations affecting the motor protein do not seem a plausible general mechanism responsible of the lack of efficacy of these compounds in the clinical setting.

Functional redundancy with other motor proteins

As mentioned in the introduction (section 1.4), there are 45 different kinesins described from which 16 have important mitotic functions. Particularly, KIF11 is an essential protein for the establishment and maintenance of the bipolar spindle and for proper chromosome segregation. However, some preclinical studies have indicated that in the absence of KIF11 other motor proteins, such as dynein or kinesin-12 (KIF15) [263,264,468,469], are able carry out its functions.

Unlike KIF11, KIF15 is not considered an essential kinesin, as it is not strictly required for bipolar spindle formation in the presence of full KIF11 activity. However, it has been demonstrated that in the absence of KIF11, KIF15 might become essential during this process. Tanenbaum and collages demonstrated that under KIF11 inhibition, ectopic overexpression of KIF15 can drive normal bipolar spindle formation. The kinesin, with the aid of the microtubule-associated protein TPX2, would generate the necessary forces to slide antiparallel MT and push centrosomes apart, avoiding the classical monopolar spindles associated to KIF11 comprised function [263]. Moreover, Sturgill *et al.* showed an alternative mechanism by which KIF15 can overtake KIF11 function that did not imply its overexpression. They proposed a novel spindle assembly pathway that involved a KIF11 rigor mutant that tightly binds to MT independently of its pharmacological inhibition or nucleotide state. KIF15 is usually located at kinetochore-MTs, where there is a high amount of MT-bundles, the preferred substrate of this kinesin. The static state of KIF11 in that context would prevent the production of motile forces generating instead high amount MT bundles. In consequence, the localization of KIF15 in the spindle would change towards non-kinetochore MT, leading to KIF15-driven spindle bipolarity [264]. Whichever the case, all authors seem to agree that KIF15 might play a crucial role in the development of resistance to KIF11 inhibitors by its ability to take over its essential functions. Based on these discoveries, it is proposed that therapeutic strategies combining the inhibition of both kinesins could help to overcome the limitations of KIF11 inhibition as monotherapy. Quite recently, it has been demonstrated that one of the few commercially available KIF15 inhibitors (KIF15-IN-1) has synergistic effects impairing tumor cell proliferation *in vitro* when combined with KIF11 inhibitors ispinesib and filanesib [255]. Nonetheless, unlike the case of KIF11, for which there are plenty of inhibitors that have been extensively characterised and tested, the development of drugs against KIF15 is in early phases and no data describing

these agents is available yet. Likewise, not clinical evidence confirming the implication of KIF15 in the development of resistance to KIF11 inhibitors has been reported.

5.5. 4SC-205 for the treatment of advanced prostate cancer

Plenty of clinical trials have been performed using KIF11 inhibitors, as discussed in section 5.4.1, however not many of them have focused on CRPC patients. A phase I trial to test the combination of ispinesib with DTX was carried out in 24 patients with advanced solid tumors, of which 14 were PCa patients. In general, no complete or partial responses were observed. Only 7 patients achieved SD (≥18 weeks), of whom 6 were CRCP. Moreover, one of these patients displayed the longest SD lasting more than 24 weeks and another showed a >50% PSA decline [470]. Although not conclusive, these results might indicate that PCa patients performed higher sensitivity to the treatment compared with patients with other tumor types. Also, a phase II trial with ispinesib was carried out in 21 metastatic CRCP patients previously treated with taxanes. In this case no responses were observed and the median PFS was only 9 weeks [471]. Authors attributed these negative results to low target expression in the tumors observed by IHC staining of specimens. However, the analysis was performed only in primary tumors, and not in metastatic lesions, which based on our experience usually express higher levels of the kinesin. Also, it is worth mentioned that analysis of circulating blood cells showed absence of monopolar spindles, which are a classical marker of KIF11 inhibitors activity [447], indicating a possible inactivity of the drug in this study. Nevertheless, these results highlight the importance of careful selection of the study cohort, especially when working with targeted therapies.

4SC-205 is a highly potent and specific KIF11 small molecule inhibitor. Unlike other clinically tested agents this one is, to the best of our knowledge, the only one that can be orally administered. This characteristic would allow a daily dosing schedule using lower concentrations of the drug, assuring sufficient target coverage while reducing the induction of adverse events [363]. In collaboration with Dr. Segura's group, we have recently published a work showing the specificity and efficacy of 4SC-205 impairing cell viability and tumor growth in several high-risk neuroblastoma models [368]. Here, based on the presented results showing that KIF11 is upregulated in advanced PCa and that it might play a role in development of CRPC, we believe that inhibition of KIF11 using 4SC-205 may be an effective therapy for CRPC patients, who presently have very limited therapeutic options. In this worked, we performed an extensive preclinical evaluation of the effects of 4SC-205 in PCa models, including CRPC. Consistent with other studies, we showed that PCa cells exposed to 4SC-205 displayed the classic phenotypic traits of KIF11 inhibition or depletion,

characterised by a mitotic arrest that eventually leads to cell death by apoptosis [316], confirming the specificity of the inhibitor for its target. Also, using a panel of six PCa cell lines, we demonstrated that 4SC-2045 was able to effectively impair cell proliferation in all of them at a nanomolar concentration range. The fact that the agent was able to similarly affect cell lines with a wide range of different molecular and phenotypic characteristic suggests that the inhibitor may be effective in a broad spectrum of PCa tumors. This fact is especially important given the highly heterogeneous nature of the disease. Remarkably, RWPE-1, an immortalised non-tumorogenic epithelial prostate cell line was by far the less affected by the inhibitor, showing an IC_{50} at least three-fold higher compared with the other models. This pointed out a higher sensitivity of PCa malignant cells to KIF11 inhibition. More importantly, 4SC-205 also showed antitumor activity in CRPC patient-derived spheroids, providing higher clinical relevance, as the effects are being tested on cells directly derived from patients with advanced PCa. Moreover, 3D models are thought to more accurately reflect the translational value of *in vitro* drug efficacy studies than two-dimensional cell monolayers [381]. To further determine the effects of KIF11 inhibition on CPRC, we evaluated the efficacy of 4SC-205 impairing tumor growth on a PDOX generated from the tumor of a high risk CRPC patient. There are some reports describing the *in vivo* efficacy of KIF11 knockdown and pharmacological inhibition in PCa cell lines derived xenograft [380,472]. However, this is the first time that the antitumor activity of a KIF11 inhibitor has been tested in a PDOX model derived from a CRPC patient. In line with previous results, we observed that tumors from mice treated with 4SC-205 were significantly smaller than those from mice treated with vehicle. As expected, 4SC-205 treated tumors showed a significantly higher fraction of cells with positive staining for H3 phosphorylation compared to controls and enhanced activation of the apoptotic signalling pathway, demonstrating a specific and active effect of the drug three weeks after treatment initiation. Collectively, these results indicated that KIF11 inhibition was able to effectively inhibit growth of several PCa models, including those representatives of the most advanced stages, highlighting the potential of KIF11 as a promising therapeutic target to treat this lethal disease.

5.6. The AR as a transcriptional regulator of KIF11

The AR is a nuclear hormone receptor that after the binding with its ligands undergoes a dimerization process and is translocated into the nucleus. Once there, the AR binds to the androgen response elements located in the promoter regions of its target genes activating their expression [142]. It is well known that the AR plays a central role in PCa and in the progression towards androgen independence, stage at which cells are able to keep the AR signaling pathway active even under castrate levels of androgens. AR overexpression, mutation, splice variants constitutively active or ligand promiscuity, among others, have been proposed as possible mechanisms responsible of this evolution [141]. However, apart from the crucial role that the AR plays in this process, little is known about the underlying mechanism and downstream pathways controlled by the AR that promote the development of CRPC. The study and termination of these pathways is essential in order to unveil novel therapeutic targets to improve the clinical management of the disease at this lethal stage.

Some years ago, Wang *et al.* showed by comparing the gene expression programs of a cell model of progression from androgen dependence to independence that a transcriptional reprogramming characterized by the upregulation of M-phase cell cycle genes was taking place during this process [192]. It caught our attention that the authors described the AR as the main responsible regulator of this event. In an attempt to elucidate the mechanisms underlying the different expression profiles between both models, Wang and colleagues performed AR ChIP-on-chip assay. They observed a higher occupancy of AR binding near the overexpressed cell cycle and mitotic genes of AI cells, suggesting that the AR might be playing an important role in this gene expression shift.

During the following years, several works have confirmed this transcriptional change toward a mitotic program. For instance, Sicar and colleagues compared the transcriptomic profile of 20 CRPC frozen surgical samples with public gene-expression data from CSPC patients. In agreement with the previous report, they observed an overexpression of mitotic-phase genes [277]. More recently, Horning *et al*., elegantly demonstrated by single-cell RNA-seq that among LNCaP cells, multiple subpopulations with different sensitivity to androgens coexist. Interestingly, they found that cell subpopulations that were less dependent on these hormones also presented transcription programs enriched in cell cycle and mitotic genes [473]. In line with all these, in previous work carried out in our group, we validated for the first time that this transcriptional reprogramming was translated at protein level using high-throughput quantitative proteomics on a cell model of androgen independence progression (manuscript in preparation). All these results suggest that mitotic proteins, under the

transcriptional control of the AR, might be playing an important role during the acquisition of androgen independence and therefore, in the progression of CRPC.

Under the idea that the AR might be promoting the expression of mitotic genes contributing with the development of CRPC and based on all the evidence that we showed pointing out that KIF11 could be implicated in that process, we decided to explore if the AR is transcriptionally regulating KIF11 in PCa. Interestingly, we found a positive and significant correlation between both genes in three independent PCa datasets but not in normal prostate tissue. This is in agreement with the previous findings and suggests that the AR might be regulating KIF11 expression specifically during PCa progression. Supporting this hypothesis, we showed that KIF11 mRNA and protein levels were increased upon AR stimulation with androgens and reduced after AR knockdown or inhibition with enzalutamide, a second-generation AR antagonist. Moreover, no effects on KIF11 level were detected after AR depletion in the PCa AR-negative cell line Du145, further indicating a direct effect of the AR on KIF11 expression. Altogether, these results seem to indicate that the enhancement of PCa cell proliferation by KIF11 is dependent on its transcriptional regulation by the AR. However, we are aware that further evidence is needed to confirm a direct transcriptional regulation of KIF11 by the AR. The determination of the presence of a putative ARE on KIF11 promoter region and its subsequent confirmation through chromatin immunoprecipitation (ChIP) and luciferase reporter assays would provide more definite proofs. Supporting the fact that the AR acts as a transcriptional factor of mitotic genes during PCa progression, recent works have demonstrated that the expression of two other mitotic kinesins, KIF4A and KIF15, is also positively correlated with AR levels in PCa [262,286]. Moreover, the authors showed solid evidence of the presence of functional AREs on the promoter region of both genes and an implication of the kinesins in the development of CRPC and hormonal therapy resistance.

Very interestingly, several studies on breast cancer (BC), which as PCa is also a hormone dependent tumor, demonstrated that the expression of KIF11 and other mitotic kinesins was regulated by the ER [261,474], another member of steroid receptor superfamily. Like the AR, ER acts as a ligand-dependent transcription factor. Both share similarities in their structures and play important roles during normal organ development and in tumor progression [475]. Moreover, it has been documented that PCa cells are able to switch from AR to ER signaling pathway after ADT as a mechanism of resistance to this therapy, suggesting that both receptors may have overlapping cistromes [164]. In their work, Planas-Silva and collages demonstrated, analogously to our results, that treatment with fulvestrand, an ER antagonist used for breast cancer treatment [476], reduced KIF11 expression in two ER-positive BC cell lines and these effects were not observed on ER-negative BC cells. Moreover, estrogen

stimulation resulted in increased KIF11 expression [474]. In line with this, Zou *et al.* demonstrated that upon estrogen stimulation of the ER, the expression of several mitotic kinesins was upregulated, being KIF11 among them. Again, fulvestrant exposure was able to reverse these effects, suggesting that kinesin upregulation was ER dependent. Moreover, they showed that high expression of mitotic kinesins was strongly correlated with shorter relapse-free survival after hormonal therapy in BC. Knockdown of these proteins remarkably increased the sensitivity of resistant BC cells to tamoxifen, a competitive inhibitor of estrogens used for the treatment of ER-positive BC [477]. In the same work, the authors demonstrated that the observed kinesin upregulation driven by the ER was dependent on ATAD2 (also known as ANCCA), a chromatin-associated protein that was described as a coactivator of ER activity. It is noteworthy that this same protein has also been identified as a coactivator of the AR in PCa [478]. Remarkably, it was reported to be upregulated in androgen independent cells and its overexpression has been proposed as prognostic tissue biomarker of CRPC. In their work, Urbanucci and colleagues demonstrated that ATAD2, which is a regulator of chromatin dynamics [479], had locus-specific effect on chromatin opening that increased AR accessibility to specific genes promoting PCa progression [480]. Intrigued by this series of coincidences and based on the previous observed correlation between KIF11 and AR expression, we thought that if AR-regulated KIF11 expression is mediated by ATAD2, a positive correlation should also be observed between KIF11 and ATAD2. We performed an *in silico* analysis and confirmed a strong and significant correlation between both genes in PCa databases, but not in the normal prostate one (**Figure 49**). This agrees with our previous observations, reinforcing our hypothesis that the AR regulates KIF11 transcription in PCa and suggests that ATAD2 may be involved in that process.

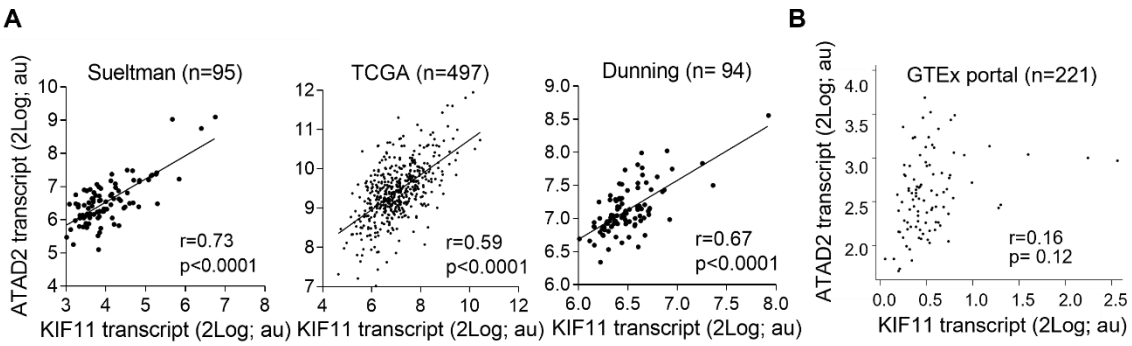

Figure 49. KIF11 expression positively correlates with ATAD2 expression in PCa but not in normal prostate tissue. (A) KIF11/ATAD2 correlation in three independent PCa datasets. **(B)** Association between KIF11 and ATAD2 expression in normal prostate samples from GTEx repository. Correlations were determined by Pearson analysis.

Altogether, these results seem to indicate that aberrant expression of ATAD2 could be a possible mechanism by which hormone dependent tumors, together with their respective steroid receptors, are able to acquire transcriptional plasticity. This would favor the expression of those specific transcriptional programs among all the functionally diverse AR targets that contribute with tumor progression, hormone independence and hormonal therapy resistance, as it has been suggested for M-phase cell cycle genes. However, this is only a hypothesis that needs to be confirmed and opens the door for an interesting future research line.

5.7. KIF11 might regulate AR transcriptional activity

One of the important questions that arises from the disappointing clinical results observed from targeted mitotic inhibitors is why classic MTA performed much better in the clinics. When first developed, microtubule targeting agents were thought to perform their antitumor activity inhibiting mitosis through MT dynamic disruption, which interferes with mitotic spindle formation resulting in cell cycle arrest and cell death [481]. However, later on it was noted that MTs also play essential roles during other phases of the cell cycle, such as intracellular transport and endocrine signalling pathways [482]. Because of that, a higher proportion of cells are susceptible to them, greatly increasing their efficacy. Nonetheless, also more severe side effects are associated with these drugs than those observed upon the treatment with targeted mitotic inhibitors, which exclusively affect dividing tissues.

Taxanes are a class of MTAs that have shown the highest survival benefit in men with CRPC compared with other chemotherapeutic agents [109]. Several works have described that the antitumor effect exert by these drugs is not only due to their antimitotic activity, but also it has been attributed to the disruption of other mechanisms by which MT-dependent pathways would drive tumor progression [482]. In the specific case of PCa, it has been suggested that the disruption of MT dynamics caused by taxane treatment is able to affect AR transcriptional activity [389–392,483,484]. This phenomenon, along with their inhibitory effect on dividing cells, is thought to be responsible of the antitumor efficacy perform by taxanes in CRPC. One of the first evidence pointing out to this effect of taxanes in PCa was described by Koruda and colleagues. In their work they showed that docetaxel treatment caused a reduction on AR protein levels that was accompanied by a decreased expression and secretion of PSA, an AR canonical target gene [483]. Similarly, Gan *et al*. showed that taxane treatment, apart from causing the expected mitotic arrest, was able to reduce the expression of PSA both, *in vitro* and *in vivo* PCa models [484]. Accordingly, Zhu and colleagues also showed a significant reduction of AR transcriptional activity upon taxane

treatment. Interestingly, they demonstrated that this decreased in AR signalling was driven by an impairment on AR nuclear translocation caused by the effect of these agents on the MT network [389]. Similar results were also reported by Darshan and colleagues [390]. In line with that, other TFs have also been described to traffic on MTs to reach the nucleus and carry out their functions, further supporting that the AR could depend on MTs for its nuclear translocation [386–388]. More recently, Martin and colleagues also showed that taxanes impaired AR trafficking and reduced AR protein levels in PCa models *in vitro* and *in vivo*. Remarkably, they showed that taxane treatment reduced the expression of several kinesins and proposed this event as a plausible mechanism by which, in addition to their effect on MT, taxanes can so effectively interfere with AR transport across microtubules, leading to the impairment of its function and eventually tumor suppression [392].

All these results indicate that MTAs are able to inhibit AR trafficking due to their effect on MTs dynamics, affecting AR intracellular distribution and protein levels, eventually leading to downregulation of its transcriptional activity. Based on that premise and given that these effects have been linked with altered kinesin expression [392], we decided to investigate a possible role of KIF11 in CRPC development by contributing with AR trafficking and transcriptional activity through its MT interacting function. Our results demonstrated that KIF11 inhibition and depletion decreased AR transcriptional activity and reduced AR and AR-V7 protein levels without affecting AR transcript, indicating that a post-transcriptional mechanism might be the responsible of that regulation. Moreover, we showed that the decrease on AR and AR-V7 proteins upon KIF11 depletion was due to a reduction of AR stability that led to its degradation. Oppositely, overexpression of the kinesin resulted in an increase of AR transcriptional activity, as shown by the enhance expression of its canonical target genes. Moreover, we showed that KIF11 inhibition was able to repress DHT-induced AR nuclear translocation. More importantly, the androgen-independent translocation of the constitutively active AR variant (AR-V7) was also reduced upon KIF11 inhibition. Altogether, we believe that KIF11 inhibition is able to block AR and AR-V7 nuclear translocation, interfering with their transcriptional activity and eventually leading to protein degradation.

Not so long ago, it was generally accepted that AR signalling was not required when PCa cells became castration resistant [485]. However, it is well known nowadays that AR pathway, together with the residual levels of androgens that remain after castration, play a crucial role in CRPC biology [486]. Based on that, research has been focused on the development of new drugs with the ability to inhibit that pathway. This led to identification of compounds such as enzalutamide [99], a potent AR antagonist, and abiraterone, an inhibitor of *de novo* steroid biosynthesis [95]. Although these agents represented an important advance in the treatment of CRPC, around 20-40% of patients display no initial response and among those

who respond, virtually all eventually develop resistance [487]. Multiple mechanisms have been attributed to the emergence of the resistance to AR targeted therapies. For instance, mutations affecting the AR LBD are commonly detected in patients after treatment with these agents [47,58,393]. Some of these mutations have been described to have the ability to transform antiandrogens into AR agonists, promoting AR signalling instead of inhibiting it. AR splice variants have also been implicated with AR targeted therapy resistance [146,488]. All AR-Vs share a typical structure that lacks the LBD, but retains intact transactivation and DNA binding domain, allowing them to be constantly active, even under castrate levels of androgens [489]. The best characterize is AR-V7, which displays constitutive nuclear localization and transcriptional activity. Moreover, it has been reported to have predictive value for enzalutamide resistance [490,491]. The nature of these mechanisms of resistance makes evident the need to develop novel strategies that target the activity of all kinds of AR isoforms, including its variants. Considering that, these new strategies should not be based on the conventional approaches that interfere with AR-ligand mediated activation. For instance, agents that block the AR transactivation domain [492,493] or the DNA binding domain [494] are now under investigation. Also, drugs that trigger AR degradation are also being studied [495–497]. Altogether, in this work we provided an initial mechanistic insight by which KIF11 inhibition by 4SC-205 might block AR and AR-V7 translocation to the nucleus across MT. By doing so, their transcriptional activity would be impaired, independently of their mutational status, and therefore also their oncogenic function. The potential role of KIF11 promoting AR and AR-Vs transcriptional activity is another proof pointing out a possible implication of this protein in CRPC progression and hormonal therapy resistance, once more highlighting its value as a therapeutic target for CRPC treatment.

5.8. Effect of the combination of KIF11 and AR inhibition in CRPC

We are aware of the limited success that KIF11 inhibitors have performed in clinical trials. However, we consider that the main utility of these agents may come from rational drug combinations with other compounds that can lead to selective tumour cytotoxicity and the avoidance of the development of resistance, commonly observed in monotherapy treatments. Considering all the results presented in this work, we proposed a mechanistic model by which the androgen receptor and KIF11 would form a positive feed-back loop promoting PCa progression. On the one hand, KIF11 would be supporting AR transcriptional activity participating on its nuclear translocation by its ability to crosslink and slide apart MTs, which are used as “tracks” by the AR. On the other hand, the AR would be enhancing the transcription of mitotic genes, being KIF11 among them, promoting cell

mitosis and feed-forwarding the previous step. The proposed mechanism provides a strong rationale for the development of a therapeutic strategy based on the combination of KIF11 inhibitor 4SC-205 with an AR-axis targeted therapy, which could improve the therapeutic response of CRCP patients compared with monotherapies. Under that premise, such combination would be affecting AR activity by the effect of the AR targeted therapy and also impairing its nuclear translocation by KIF11 inhibition. Moreover, mitotic progression would be also affected at two levels: by blocking spindle formation as a result of KIF11 inhibition and by the impairment of AR signalling, which has been described to preferentially promote expression of mitotic genes in CRPC.

There are no clinical data supporting neither the efficacy, nor the safe and tolerability of this kind of combination. However, based on the above-discussed results from preclinical studies showing the inhibitory effects of taxanes on AR signalling pathway, some clinical trials have been carried out in order to determine the therapeutic benefit of these agents in combination with hormonal therapy in CRPC patients. For instance, a phase Ib trial was carried out to assess safety and tolerability of docetaxel in combination with enzalutamide in mCRPC patients [498]. As the trial had not comparator arm, results were compared with other studies that used these agents as monotherapy. PSA decline ≥ 50% was observed in almost all patients (95%) with a median duration of response of 226 days. A ≥90% PSA reduction occurred in 65% patients. These response rates were higher than the observed in the TAX 327 study [499], in which 45% of patients treated with docetaxel alone performed ≥50% PSA reduction. These outcomes were also better than those observed for patients treated with enzalutamide as monotherapy in the PREVAIL phase III trial [397], in which 78% of patients displayed ≥50% reduction of PSA. Related with the safety and tolerability of the combination, neuropathy was the most common cause that forced docetaxel treatment discontinuation. No case was assessed to enzalutamide treatment. A total of 16 patients over 22 (73%) reported signs of any grade peripheral neuropathies. High grade neutropenia was also a highly common adverse event (86.4%). As it was a nonrandomised study with a small sample size and without comparator arm, PSA outcomes should be carefully interpreted. However, the results were considered promising and led to the initiation of other studies with a bigger population. Very recently, results from the first phase II randomised trial (CHEIRON study) evaluating the efficacy of the combination of docetaxel and enzalutamide in mCRPC patients were published [500]. The combination showed superiority compared to docetaxel alone in terms of ≥50% PSA reduction (94% *vs*. 74%, respectively) and disease-free rates after six months (88%% *vs*. 62%, respectively). Remarkably, visceral metastasis showed complete or partial response in 86% of patients treated with the combination regimen compared to 50% in docetaxel monotherapy group. Again,

haematological related toxicities were common adverse events and were more frequent in the combination arm. Incidence of neuropathies was equal in both groups. Altogether, these results suggest that the combination of both agents may be more clinically beneficial for the treatment of mCRPC patients than any drug as monotherapy. Although promising, bigger studies are needed to confirm these results and to compare the efficacy of the combination against enzalutamide alone.

The preclinical and clinical studies previously discussed suggest that the combination of taxanes with antiandrogens could perform improved therapeutic results in CRPC patients compared to single agents. Given that we have demonstrated that KIF11 inhibition is able to mimic the inhibitory effects performed by taxanes on AR transcriptional activity, we decided to test the effect of the combination of the antiandrogen enzalutamide with KIF11 inhibitor 4SC-205. Our findings showed that the combination of both drugs have strong synergistic effects impairing cell viability of two CRPC models *in vitro*. More importantly, using a transgenic mouse model that displays molecular and phenotypic characteristics of CRPC patients, we demonstrated that the combination regime significantly inhibited tumor growth *in vivo* compared to any of both drugs as monotherapy. Thus, we believe that the combination of 4SC-205 and enzalutamide could improve disease control of CRPC patients compared to monotherapies in a similar way as it has been described for taxanes and enzalutamide, adding some other clinical benefits. For instance, the severe side effects associated with these agents in terminally differentiated cells, such as neurons, would be avoided. In addition, the characteristic oral administration of 4SC-205 that allows a low dose daily regime would ensure more constant target coverage, overcoming the proliferation rate paradox without drastically increasing the DLT. This, along with the use prophylactic bone marrow stimulating cytokines, could provide a wider therapeutic window and eventually lead to improved response rates.

In summary, the findings described in this thesis provide interesting contributions in the field of targeted therapies in PCa, and more particularly CRPC. From a preliminary proteomic analysis of a model of androgen independence progression we obtained a list of interesting mitotic candidates potentially related with this process. Among all candidates, KIF11 outstood, being selected for further analysis in that field. We were able to detect several indications relating this protein with CRPC that highlighted its potential value as a predictive biomarker and therapeutic target for this lethal disease. Although further research is needed, our findings provide initial insights about a possible role of that motor protein in CRPC development by its ability to modulate AR signalling in PCa. Moreover, the AR would be feed-forwarding the whole process, as well as cell proliferation, modulating KIF11 transcription. Altogether, these results may help to develop future rational therapeutic

strategies based on the dual inhibition of the AR and KIF11 with the potential to increase response and survival rates of CRPC patients avoiding the use of current compounds associated with highly severe side effects.

6. Conclusions

First: KIF11 is upregulated in (I) androgen independent PCa cell lines compared with the androgen dependent one and (II) in CRPC samples compared to benign tissues, localized hormone naïve PCa and to mCSPC. Moreover, KIF11 overexpression in a PCa AD model promotes its proliferation in androgen depleted conditions, altogether indicating a potential role of this protein in the acquisition of castration resistant characteristics.

Second: High levels of KIF11 are correlated with more aggressive histological grades, biochemical recurrence, shorter response to hormonal-treatment and worse progression free survival, suggesting that KIF11 could be a good prognosis marker and therapy response predictor for PCa patients.

Third: KIF11 pharmacological inhibition with 4SC-205 causes a mitotic arrest with accumulation of cells in G2/M phase, eventually leading to cell death via apoptosis.

Fourth: 4SC-205 impairs PCa cell growth *in vitro*, decreases cell viability of CRPC patient-derived 3D cultures *ex vivo* and significantly reduces tumor growth of a CRPC PDOX *in vivo,* highlighting the value of KIF11 as a novel therapeutic target for the treatment of CRPC.

Fifth: KIF11 protein levels are decreased upon AR depletion or inhibition with enzalutamide in PCa AR-positive cell lines but not in the AR-negative one. Also, KIF11 protein and mRNA expression are increased upon AR stimulation, suggesting a possible transcriptional regulation of KIF11 by the AR.

Sixth: KIF11 downregulation causes a decrease on AR and AR-V7 protein levels and reduces the expression of AR canonical target genes, but not AR transcript. Furthermore, KIF11 inhibition impairs AR and AR-V7 nuclear translocation and reduces their protein stability, suggesting that KIF11 might modulate AR transcriptional activity participating on its nuclear trafficking.

Seventh: Dual inhibition of KIF11 and the AR axis synergistically inhibits the growth of CRPC models both, *in vitro* and *in vivo*, and provides a good rationale for a combination therapy using 4SC-205 and enzalutamide for the treatment of CRPC patients.

7. Publications

During the course of this thesis, several works and collaborations have been performed giving place to the following publications:

1. Rapado-González Ó, Majem B, Álvarez-Castro A, Díaz-Peña R, Abalo A, **Suárez-Cabrera L**, Gil-Moreno A, Santamaria A, López-López A, Muinelo-Romay L, Suarez-Cunqueiro MM. A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis. J Clin Med. 2019 Nov 20;8(12).

2. Majem B, Parrilla A, Jiménez C, **Suárez-Cabrera L**, Barber M, Marín A, Castellví J, Tamayo G, Moreno-Bueno G, Ponce J, Matias-Guiu X, Alameda F, Romero I, Sanchez JL, Perez-Benzavente A, Morán S, Esteller M, Reventós J, Rigau M, Gil-Moreno A, Segura MF, Santamaria A. MicroRNA-654-5p suppresses ovarian cancer development impacting on MYC, WNT and AKT pathways. Oncogene. 2019 Aug 5;38(32):6035–50.

3. Masanas M, Masiá N, **Suárez-Cabrera L**, Olivan M, Soriano A, Majem B, et al. The oral KIF11 inhibitor 4SC-205 exhibits antitumor activity and potentiates standard and targeted therapies in primary and metastatic neuroblastoma models. Clin Transl Med. 2021;11(10):e533.

4. Olivan M, Garcia M, **Suárez-Cabrera L**, Guiu M, Gros L, Méndez O, Rigau M, Reventós M, Segrua MF, de Torres I, Planas J, de la Cruz X, Gomis RR, Morote J, Rodríguez-Barrueco R, Santamaria A. Loss of microRNA-135b Enhances Bone Metastasis in Prostate Cancer and Predicts Aggressiveness in Human Prostate Samples. Cancers (Basel). 2021 Dec 9;13(24).

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