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Decoding androgen receptor inhibitor resistance in prostate cancer

Sander Anton Leopold Palit

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Het ontrafelen van androgeenreceptor remmer resistentie in prostaatkanker

(met een samenvatting in het Nederlands)

Proefschrift

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Sander Anton Leopold Palit

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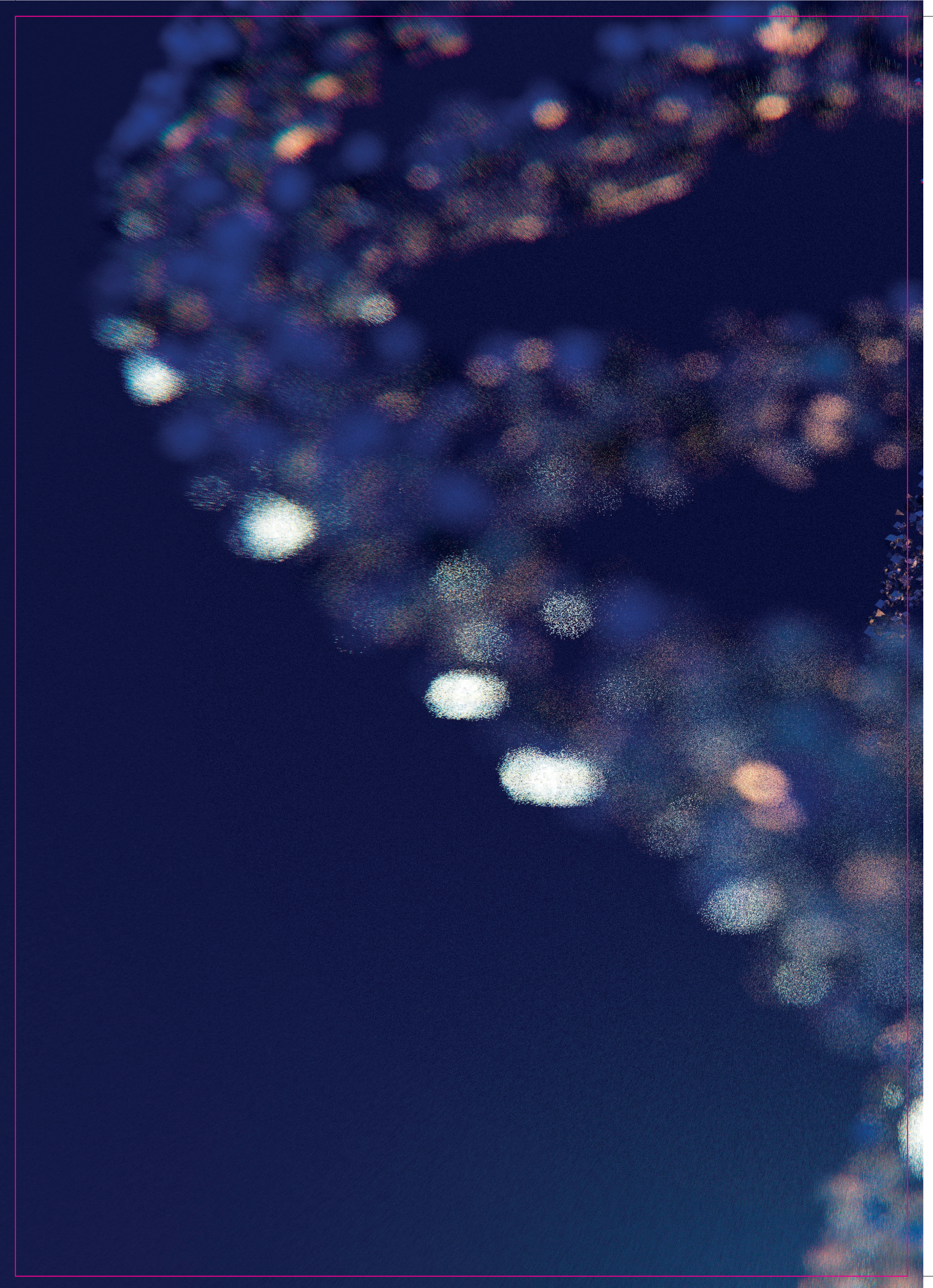

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Chapter 1

General introduction

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Prostate cancer diagnosis and treatment

Prostate cancer is the second most frequent form of cancer diagnosed in men, with worldwide over 1.3 million new cases and around 359,000 cancer-related deaths each year (Bray et al., 2018). At present, the diagnosis of prostate cancer is based on the assessment of histological features of the tumor (Gleason score), imaging technology, and the levels of prostate-specific antigen (PSA) in the serum of the patient. The Gleason score, which is a measure reflecting tumor aggressiveness, is determined through microscopic evaluation of needle biopsy material by a pathologist. Prostate tumors typically consist of distinct populations of tumor cells with varying histological grades. The Gleason score is based on the sum of two numbers (each on a scale from 1 to 5) describing the histological patterns of cancerous cells within the tumor. The higher the number, the more abnormal and poorly differentiated the cells are. The first number describes the most predominant histological pattern observed, and the second number describes the second-most predominant pattern present in the examined tissue. Combined, the two numbers make up the Gleason score (e.g. Gleason 3 + 4 = 7). The combined data of the PSA levels, Gleason score and clinical stage are used to stratify patients into one of three risk groups; low, intermediate or high risk (Litwin & Tan, 2017).
Based on their risk group, patients with localized prostate cancer typically have 3 options: watchful waiting/active surveillance, surgery and radiation. Watchful waiting centers around palliative care and managing symptoms, while active surveillance assesses disease progression using various methods including prostate biopsies, physical examinations and measuring PSA levels. Active surveillance allows for selection of patients who develop significant disease that requires treatment. When the cancer is confined to the prostate gland, it can often be treated with local intervention involving surgery and/or radiation with curative intent. Unfortunately, after treatment, recurrence of prostate cancer is observed in about 30% of cases, which is characterized by metastatic disease that is incurable (Litwin & Tan, 2017).

Prostate cancer preferentially metastasizes to the bone, observed in nearly 90% of patients. Other common sites of metastasis are the lymph nodes (LN), liver and visceral sites (Bubendorf et al., 2000; Budczies et al., 2015; Gandaglia et al., 2015; Shou et al., 2018). The site of prostate cancer metastasis is associated with overall survival and cancer-specific survival. Overall survival is highest in patients with exclusively LN metastases, followed by bone-only metastases (Gandaglia et al., 2015; Pond et al., 2014). Patients with liver metastases or visceral lesions, with the exception of lung metastases, show worse 5-year overall survival, which exacerbates when concurrent bone metastases are present (Gandaglia et al., 2015; Halabi et al., 2016; Halabi et al., 2014). Collectively, these studies show that the presence and location of the metastatic site

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strongly associates with patient survival.
Cancer metastasis occurs through a non-stochastic and cancer type-specific process termed "metastatic organotropism", governed by tumor-microenvironment interactions at the pre-metastatic niche (Gao et al., 2019; Liu & Cao, 2016). Tumor cell-intrinsic factors that influence metastatic organotropism include tumor cell growth and survival signals (Jacob et al., 2015), altered metabolism (Pani et al., 2010; Webber et al., 2010), and the expression of cell surface markers and secreted factors that may prime tumor cells, as well as the pre-metastatic niche, for dissemination to specific tissues (Barthel et al., 2013; Peinado et al., 2011). Metastatic colonization is further influenced by the vascular architecture of tissues and the biochemical milieu of the stroma, which may either impede or facilitate engraftment of metastasizing tumor cells (Smith et al., 2017). The process of metastatic organotropism in prostate cancer is still poorly understood. More insight into the underlying mechanisms of this process and key genetic determinants that control metastatic organotropism may improve patient stratification and facilitate the identification of cellular processes amenable for therapeutic targeting to improve patient survival.

The vast majority of prostate cancers are hormone driven, specifically through deregulated androgen signaling. Therefore, the mainstay of systemic treatment for advanced prostate cancer centers around suppressing androgen signaling through androgen deprivation therapy (ADT), which lowers the levels of androgens in the serum of patients to castrate levels (<50 ng/dL). Commonly used drugs for ADT are gonadotropin releasing hormone (GnRH) agonists such as leuprolide and goserelin (Crawford et al., 2019; Perlmutter & Lepor, 2007). GnRH agonists function by binding to the GnRH receptor, leading to the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. Continued activation of GnRH signaling by GnRH agonists results in GnRH receptor downregulation, shutting down the pathway. As a result, the production of gonadal testosterone is halted, pushing the levels of androgens in the blood towards castrate levels (Perlmutter & Lepor, 2007). Blockage of androgen signaling by ADT is effective in suppressing prostate tumor growth in the clinic, and can bring patients in remission for up to several years (mean time of 2-3 years) (Karantanos et al., 2013). However, tumors inevitably develop mechanisms allowing them to resume androgen signaling despite castrate-levels of androgens in the serum. At this stage, the disease is referred to as castration-resistant prostate cancer (CRPC). Even though CRPC is able to grow despite castrate-levels of androgens, most of these tumors still depend on this pathway and its key component, the androgen receptor (AR) (Crawford et al., 2019; Karantanos et al., 2013).

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Genetic hallmarks of advanced prostate cancer

The androgen receptor (AR, gene symbol *NR3C4*) is a major driver in CRPC (Robinson et al., 2015; Taylor et al., 2010). AR belongs to the family of steroid hormone receptors (SHR) which include the estrogen receptor (ER), progesterone receptor (PR), the mineralcorticoid receptor (MR) and the glucocorticoid receptor (GR). The *AR* gene itself is 90 kilobases (kb) in length, encoding for a protein with a molecular weight of 110 kilodalton (kDa). Like the other members of the SHR family, AR comprises three major functional domains: the N-terminal domain (NTD), DNA-binding domain (DBD) and ligand-binding domain (LBD). The NTD of AR plays a critical role in the transactivation activity of the receptor. It was shown to bind a variety of transcription factors implicated in gene regulation, such as TATA-box binding protein (TBP), transcription factor IIF (TFIIF), P160 coactivator proteins, and the histone acetyl transferase CREB-binding protein (CBP) among others (Jimenez-Panizo et al., 2019; Tan et al., 2015).
The DBD region of AR is highly conserved. It is responsible for the binding of the receptor to DNA elements known as enhancers. These enhancers consist of two half-sites, each comprising six nucleotides (5'-AGAACA)-3'), that are separated by 3 base-pair spacer. Variations in these sequences contribute to SHR binding specificity for different SHRs at distinct enhancer elements. For example, AR-specific enhancers, termed androgen response elements (ARE), have a specific half-site sequence (5'-GGTTCT-3') (Claessens et al., 1996; Jimenez-Panizo et al., 2019; Shaffer et al., 2004; Tan et al., 2015).
The DBD is connected to the LBD by the hinge region, which contains the nuclear localization signal (NLS) that facilitates translocation of the receptor from the cytoplasm to the nucleus upon androgen binding. The LBD contains the ligand binding pocket which binds AR ligands resulting in activation of the receptor. Therefore, most drugs that are used in the clinic for systemic treatment of prostate cancer that target the androgen receptor are directed towards this region of the protein (Jimenez-Panizo et al., 2019; Tan et al., 2015).
In the absence of androgens, AR resides dormant in the cytoplasm where it is bound by chaperone proteins, such has heat-shock protein 90 (HSP90). Upon binding of androgens, AR undergoes significant conformational changes and dissociates from the chaperone proteins that sequester it in the cytoplasm. Consequently, AR utilizes the NLS to translocate to the nucleus where it dimerizes and binds to its cognate enhancers on the DNA. Binding of AR to its enhancer is facilitated by pioneer transcription factors that function to create a chromatin structure that is permissive for AR binding. Once bound to the enhancer, AR recruits a variety of transcription factors to form a protein complex that regulates the expression of target genes (Jimenez-Panizo et al., 2019; Tan et al., 2015). AR predominantly binds distal enhancers in intergenic regions, typically located >20 kb from the transcription start site (TSS) of its target genes. A single gene can be

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under the control of several AR enhancers. Upon assembly of the AR complex, consisting of the receptor and its co-factors, the complex interacts with the target gene to either promote or inhibit gene expression (Stelloo et al., 2019). Figure 1 shows a schematic representation of AR-mediated gene regulation, and some notable AR-directed therapeutics and where they impinge on the AR pathway.

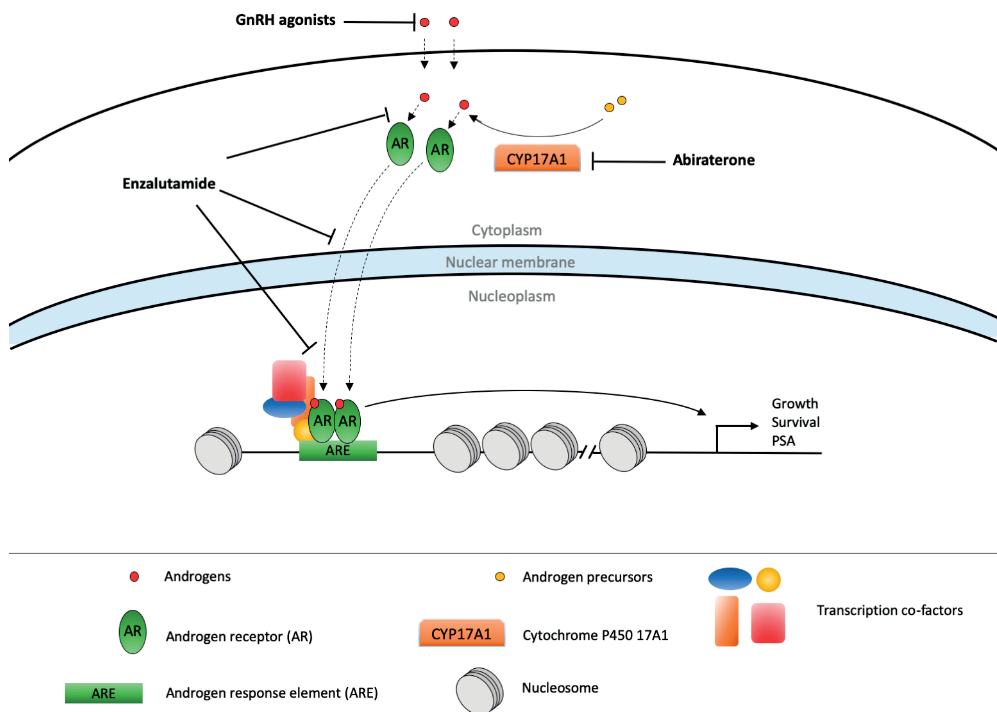

Figure 1: The AR pathway and cornerstone therapeutics in prostate cancer.
AR resides in the cytoplasm and translocates to the nucleus upon androgen binding. In the nucleus AR binds distal enhancers termed androgen response elements (ARE). There, AR recruits transcription co-factors to regulate transcription of target genes. GnRH agonists suppress the production of gonadal androgens by downregulating the expression of the GnRH receptor. Abiraterone inhibits CYP17A1, which is implicated in the conversion of intratumoral androgen precursors to androgens. Enzalutamide is an AR antagonist, it binds AR thereby preventing the binding of androgens (competitive binding). Once bound, enzalutamide renders the receptor inactive, preventing its translocation to the nucleus, binding to enhancers and recruitment of transcription co-factors required for transactivation.

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In the normal prostate, AR functions to maintain prostate homeostasis by regulating the expression of genes involved in prostate development and cell differentiation. In CRPC, AR expression and activity is altered. AR signaling is re-wired as a result of genetic and epigenetic alterations that change the AR cistrome and transcriptional program, resulting in deregulation of genes involved in cell growth and survival (Zhou et al., 2015). For example, various genetic aberrations affecting transcriptional co-factors that collaborate with AR, resulting in altered activity of the receptor, have been described and are a hallmark of prostate tumorigenesis. Insight into the role of AR transcriptional co-factors is essential to understanding prostate cancer biology and therapy response.
One such transcription factor is homeobox B13 (HOXB13). HOXB13 belongs to the conserved homeobox family of transcription factors implicated in gene regulation, and has a central role in the development of the male reproductive tract (Brechka et al., 2017). Mutations in this gene, although less frequent, have been reported for prostate cancer, most notably the germline G84E mutation which is associated with increased prostate cancer risk (Ewing et al., 2012). Deregulation of HOXB13 binding at enhancers was previously associated with an oncogenic AR cistrome, making it a key player in prostate tumorigenesis. HOXB13 was shown to colocalize with FOXA1 at tumor-specific AR enhancers where they collaborate to reprogram the AR cistrome (Pomerantz et al., 2015; Stelloo et al., 2018). This was confirmed by the characterization of the endogenous AR protein interactome, which revealed an AR subcomplex comprising AR, FOXA1 and HOXB13 involved in prostate tumorigenesis. These findings are consistent with the increased expression of HOXB13 in tumor tissue compared to normal tissue (Stelloo et al., 2018).
Forkhead box protein A1 (FOXA1) deregulation is a well-known driver of prostate cancer development and progression that has been the focus of intense research (Barbieri et al., 2012; Gerhardt et al., 2012; Sahu et al., 2011). FOXA1 functions as a pioneer factor that facilitates AR recruitment to enhancers thereby co-regulating gene expression. In prostate cancer, FOXA1 is recurrently mutated and various alterations have been identified, with most mutations found in the Forkhead DNA binding domain affecting the interaction of the protein with the DNA and nuclear movement (Parolia et al., 2019; Teng et al., 2021). Thus, in addition to collaborating with HOXB13 to reprogram the AR cistrome, FOXA1 can be mutated effecting divergent changes in AR binding and transcriptional activity, contributing to prostate cancer (Teng et al., 2021). FOXA1 expression was shown to be associated with CRPC, AR signaling, tumor progression and invasion, with high levels of FOXA1 being associated with rapid biochemical relapse (Gerhardt et al., 2012; Robinson et al., 2014; Stelloo et al., 2018; Tsourlakis et al., 2017). However, conflicting results have also been reported (Jin et al., 2013; Wang et al., 2011), illustrating the importance of proportionate FOXA1 expression levels, and the need to dissect the mechanisms that dictate oncogenic FOXA1 function in this context.

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Gene fusions involving the AR target gene *Transmembrane Protease Serine 2* (*TMPRSS2*) and members of the ETS transcription factor family are commonly found in prostate cancer, most notably the *TMPRSS2-ERG* fusion which is found in ~50% of prostate cancer cases (Iljin et al., 2006; Soller et al., 2006; Tomlins et al., 2006; Tomlins et al., 2005). ETS transcription factors regulate genes implicated in differentiation and proliferation, contributing to tumorigenesis when deregulated (Sizemore et al., 2017). As a result of fusions with the *TMPRSS2* gene, expression of ETS transcription factors comes under the aberrant control of AR, changing the AR-mediated transcriptional output of these cells. Important to note is that expression of TMPRSS2-ERG alone is insufficient for the malignant transformation of cells (Carver et al., 2009; Clark & Cooper, 2009; King et al., 2009), and its functional role in prostate tumorigenesis is still poorly understood. Several studies have provided evidence implicating collaboration between TMPRSS2-ERG fusions and loss of phosphatase and tensin homologue (PTEN) expression (King et al., 2009), the latter being observed in up to 50% of CRPC cases (Jamaspishvili et al., 2018). The co-occurrence of PTEN loss and TMPRSS-ERG expression in malignant cells suggests that these two genetic events work together in the malignant transformation leading to prostate cancer. This is supported by studies in mice showing that PTEN loss and TMPRESS-ERG collaborate in prostate tumorigenesis (Carver et al., 2009; King et al., 2009). Using a conditional mouse model, it was shown that concurrent PTEN loss and TMPRSS2-ERG expression resulted in an invasive prostate cancer phenotype, which was accompanied by changes in the AR cistrome mediated by ERG (Chen et al., 2013). Collectively, these studies show that prostate cells may be primed for malignant transformation by PTEN loss or TMPRSS2-ERG expression, while co-occurrence appears to promote progression to prostate cancer.

Expression of the tumor suppressor PTEN is frequently lost in various cancers, and is one of the major mechanisms resulting in activation of the phosphatidylinositol-3-kinase (PI3K) pathway, a major cell growth and proliferation pathway. Genetic alterations leading to PI3K pathway activation are found in around 40% of primary prostate cancers and are almost invariably present in advanced disease (Robinson et al., 2015; Taylor et al., 2010). Moreover, the PI3K pathway was also shown to interact with the AR pathway in a reciprocal manner, where inhibition of one pathway activates the other (Carver et al., 2011). This reciprocal feedback, and the prevalence of activated PI3K signaling, predominantly through PTEN loss, in advanced disease illustrates the importance of the PI3K pathway in the transition to the androgen-independent state and poor response to AR-directed drugs. Increasing evidence revealing the interplay between the PI3K and AR pathway suggest that inhibitors targeting these pathways may interchangeably influence their activity affecting therapy response and tumor evolution, providing the rationale for co-targeting of the PI3K and AR pathway (Carver et al., 2011; Crumbaker et al., 2017).

Another major signaling pathway that is deregulated in prostate cancer is the Ras/Raf/

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MEK/ERK pathway, also known as the MAPK pathway which is involved in various cellular processes including cell proliferation and survival. Alterations in the MAPK signaling pathway are observed in up to 40% of primary and 90% of metastatic cases (Robinson et al., 2015; Taylor et al., 2010), pointing towards a role for oncogenic MAPK signaling in prostate cancer progression to the androgen-independent state. Even though members of this pathway are rarely mutated in prostate cancer, amplifications of MAPK-related genes are more frequently observed in CRPC (Robinson et al., 2015; Taylor et al., 2010). The fact that the PI3K and RAS pathways are both more activated in advanced disease, points towards their role in the progression of these tumors towards androgen-independent growth, and emphasizes their importance as therapeutic targets in mCRPC.

Loss of tumor suppressor retinoblastoma (RB1) function through alterations in the *RB1* gene itself or interactors of RB1, are associated with dissemination of the disease and progression to CRPC, and a poor clinical outcome (Robinson et al., 2015; Taylor et al., 2010; Thangavel et al., 2017). RB is phosphorylated by cyclin-dependent kinases (CDKs) which are regulated in a cell cycle-dependent fashion. In the unphosphorylated state, RB binds and thereby inactivates the E2F transcription factor, inhibiting G1/S cell cycle progression by repressing E2F target gene activation. Complete phosphorylation of RB (p-RB) results in its dissociation from E2F, allowing E2F-mediated transactivation of target genes implicated in cell cycle progression. Impaired RB function results in aberrant activation of genes regulated by E2F, resulting in uncontrolled cell cycle progression and growth (Dick & Rubin, 2013; Giacinti & Giordano, 2006). Importantly, it was shown that AR signalling is directly regulated by RB1 (Sharma et al., 2010). Thus, the role of RB loss of function in the progression towards the castrate-resistant state is reflected by the increased frequency of alterations impinging on RB and therapeutic failure in later stages of the disease.

Finally, prostate tumorigenesis is characterized by genetic alterations affecting *AR* itself, with *AR* alterations being found in about half of primary prostate cancer cases and in virtually all metastatic cases, illustrating the importance of this receptor in disease progression and therapy response, making it a hallmark of prostate cancer (Robinson et al., 2015; Taylor et al., 2010; van Dessel et al., 2019). Both genetic and epigenetic mechanisms may lead to AR upregulation in CRPC. For example, amplification of the *AR* gene as well as the upstream enhancer that regulates *AR* expression are found in most clinical samples of advanced disease and are associated with therapeutic response to AR-directed therapies and survival (Dang et al., 2020; Porter et al., 2021; Robinson et al., 2015; Takeda et al., 2018; Taylor et al., 2010; Viswanathan et al., 2018). The central role of AR and it's collaborating factors in advanced prostate cancer and therapy response, emphasize the importance of a better mechanistic understanding of this signaling pathway for the development of novel treatment avenues that will benefit patients.

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Targeting the androgen receptor in prostate cancer

Due to the pivotal role of AR in driving CRPC, compounds that target the receptor are the cornerstone drugs for the treatment of advanced disease. Two drugs that are used in the clinical setting for the treatment of CRPC are abiraterone and enzalutamide (Beer et al., 2014; Ryan et al., 2013; Tran et al., 2009). A drug that was added to the list more recently is apalutamide, a compound similar to enzalutamide that works in the same mode of action (Chi et al., 2019; Smith et al., 2018). Abiraterone is a potent and selective inhibitor of the enzyme cytochrome P450 17A1 (CYP17A1). Binding of abiraterone results in the irreversible inhibition of the enzyme. CYP17A1 catalyzes the conversion of hormones, such as progesterone and pregnenolone, to androgen precursors such as androstenedione and dehydroepiandrosterone, leading to the biosynthesis of androgens including testosterone which ultimately activate AR to drive tumor growth (Fig. 1) (Ryan et al., 2013). Expression of CYP17A1 is often upregulated in CRPC, thereby contributing to tumor growth in AR-driven prostate cancer. The efficacy of abiraterone was demonstrated in clinical trials, showing clear superiority over placebo in CRPC patients who had progressed on or after docetaxel (de Bono et al., 2011) and in patients who had no yet received systemic therapy (Ryan et al., 2013) (Fig. 2A). The median radiographic progression-free survival in the trial by Ryan et al. (2013) for patients receiving abiraterone and prednisone was 16.5 months, which was 8.3 months for patients receiving prednisone alone. Furthermore, the overall survival improved for patients receiving abiraterone relative to patients of the control group (Ryan et al., 2013).

Another therapeutic agent that has demonstrated efficacy in CRPC is enzalutamide. Enzalutamide is an AR antagonist that directly binds the LBD of the receptor. Binding of enzalutamide inhibits AR activity through competition with other androgens. Importantly, once enzalutamide has bound AR it prevents nuclear translocation, dimerization, interaction with the DNA and recruitment of transcriptional co-factors. Thus, enzalutamide acts on several nodes of the AR pathway, rendering the receptor inactive, thereby inhibiting signaling and downstream cellular processes involved in tumorigenesis (Fig. 1) (Beer et al., 2014; Tran et al., 2009). Enzalutamide showed superiority over placebo in patients with and without prior chemotherapy treatment (Azad et al., 2015; Beer et al., 2014). Patients treated with both enzalutamide and ADT showed significantly reduced risk for metastatic progression compared to patients treated with ADT alone (Armstrong et al., 2019). For the study by Beer et al. (2014), the median radiographic progression-free survival was not reached for patients receiving enzalutamide in this study, while for patients receiving placebo this was 3.9 months (Fig. 2B).

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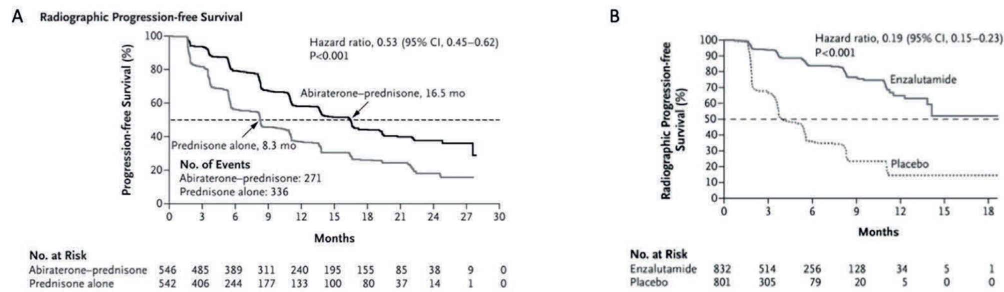

Figure 2: The radiographic progression-free survival of prostate cancer patients receiving abiraterone, enzalutamide or placebo. (A) Radiographic progression-free survival in patients treated with or without abiraterone (Ryan et al., 2013). (B) The therapeutic response determined by radiographic progression-free survival is shown for patients treated with enzalutamide or placebo (Beer et al., 2014).

In this study, patients receiving enzalutamide had a reduced risk (81%) of radiographic progression or death when compared to patients receiving placebo after twelve months (Beer et al., 2014). The efficacy of AR-directed drugs such as abiraterone and enzalutamide demonstrate the importance of resumed AR signaling in CRPC and the dependence of these tumors on AR activity. However, patients invariably develop resistance to these drugs and eventually succumb to the disease, illustrating a pressing need for a better mechanistic understanding of CRPC and therapeutic resistance to current treatments.

Therapeutic resistance to enzalutamide in prostate cancer

Enzalutamide resistance remains a problem in the treatment of advanced prostate cancer. Resistance to enzalutamide may be the result of primary (intrinsic) resistance, where tumors are insensitive to the drug at treatment onset (Buttigliero et al., 2015). Primary resistance to enzalutamide occurs in about 10% to 20% of prostate cancer patients, with patients that are pre-treated with chemotherapy showing a worse response compared to chemotherapy-naïve patients (Beer et al., 2014). Primary resistance, although occurring in a significant proportion of patients, is relatively under-examined, and research focusing on gaining mechanistic insight into primary enzalutamide resistance and novel therapeutic approaches for this subset of patients could significantly improve prostate cancer patient care (Buttigliero et al., 2015). In most cases, however, treatment with enzalutamide is characterized by an initial response illustrated by tumor regression followed by acquired resistance and resumed tumor growth (Beer et al., 2014). Acquired resistance has been the focus of intense research, and various underlying mechanisms have been proposed.

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For example, mutations in AR have been associated with enzalutamide resistance. These mutations typically affect the LBD of AR, where enzalutamide exerts its function. One example is the F877L mutation in AR, leading to a phenylalanine to leucine substitution at positing 877, which was shown to confer enzalutamide resistance *in vitro* and *in vivo* (Joseph et al., 2013; Korpal et al., 2013). The T878A mutation, leading to a threonine to alanine substitution, was shown to require the F877L mutation in conferring full agonistic function of AR to enzalutamide in this context. Although infrequent, F877L and T878A double mutants have been identified in patients where they were associated with enzalutamide resistance (Azad et al., 2015; Prekovic et al., 2018). However, it must be noted that the low frequency of clinically relevant AR mutations indicates that genetic mutations in this gene are not a common mode of resistance employed by prostate cancer cells to overcome enzalutamide treatment (Azad et al., 2015).

Prostate cancer cells are known to express various splice variants of AR, which were shown to predominantly arise during enzalutamide treatment in both pre-clinical models as well as patient samples (Antonarakis et al., 2014; Guo et al., 2009; Hu et al., 2009; Li et al., 2013). Interestingly, most of the AR splice variants lack the LBD to a varying degree. Their increased expression upon antihormonal therapy and the retained ability for some of the splice variants to regulate transcription while lacking the LBD (the region where enzalutamide binds to antagonize AR), has sparked great interest in these splice variants, specifically regarding their potential role in prostate cancer progression and therapy response (Wadosky & Koochekpour, 2017). The AR-V7 (AR3) splice variant has been studied the most in this context. Hu et al. (2009) established AR-V7 as the most predominant AR-V and found it to be the most widely and endogenously expressed at protein level among examined *in vitro* models in their study. AR-V7 lacks the LBD and contains only exons 1, 2, and 3 together with a cryptic exon 3b. Despite loss of the LBD, AR-V7 was shown to be able to bind DNA and effect transcription, activating a distinct transcriptional program (Li et al., 2013). Moreover, AR-V7 was shown to be present in the nucleus in the absence of androgens, suggesting constitutive activity (Guo et al., 2009; Li et al., 2013). A study that examined longitudinal AR-V7 expression in circulating tumors cells (CTC) from patients revealed that expression of AR-V7 correlated with a poor response to enzalutamide treatment (Antonarakis et al., 2014). Several clinical trials focus on the prospect of AR-V7 as a predictive biomarker for the response to AR-directed therapies (Wadosky & Koochekpour, 2017).

Intracrine androgen biosynthesis is another mechanism utilized by prostate cancer cells to resume growth despite anti-hormonal treatment (Adeniji et al., 2013). Upregulation of the enzyme aldo-keto reductase family 1 member C3 (AKR1C3), which catalyzes the conversion of androgen precursors to testosterone and DHT, has been implicated in

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the intratumoral production of androgens in prostate cancer cells. Overexpression of AKR1C3 is enriched in patients with enzalutamide-resistant CRPC. Studies in pre-clinical models of prostate cancer revealed that AKR1C3 is upregulated in response to antihormonal treatment, and that inhibition of this enzyme potentiates enzalutamide efficacy. Therefore, AKR1C3 may represent an appealing drug target for CRPC patients treated with enzalutamide, or patients harboring tumors that have developed resistance to enzalutamide (Adeniji et al., 2013).

Lineage switching represents another mechanism resulting in enzalutamide resistance. In cancer biology, lineage plasticity refers to the capability of cancer cells to switch back to cell lineages with distinct morphology and divergent characteristics and traits (Ku et al., 2017; Mu et al., 2017). This mechanism may allow cells to alter drug targets in a way that facilitates the evasion of the therapeutic. For example, in the case of enzalutamide resistance, it involves the ability of prostate cancer cells to switch from AR-dependent, to becoming AR-independent. Lineage switching in prostate cancer centers around the deregulation of several transcription factors and their ability to alter the epigenome leading to AR-independence and increased expression of stem cell and neuro-endocrine markers. In this context, loss of TP53 and RB1 leads to an increase in SOX2 expression altering its activity, thereby deregulating genes that drive the shift in cell stemness and functional phenotype from AR-dependent to AR-independent conferring drug resistance (Adeniji et al., 2013; Ku et al., 2017).

Emergence of neuroendocrine prostate cancer (NEPC) is purported to be driven by antihormonal treatment of prostate cancer, leading to clonal selection of NEPC therapy resistant cells originating from prostate adenocarcinoma (Patel et al., 2019) (Clermont et al., 2019). NEPC is an aggressive form of prostate cancer characterized by its metastatic potential. NEPC is reported to be present in up to 20%-30% of CRPC cases (Patel et al., 2019)(Clermont et al., 2019), illustrating its clinical relevance. Important to note is that NEPC is AR-negative and does not express PSA. NEPC is characterized by genetic aberrations which result in loss of tumor suppressors *TP53*, *PTEN* and *RB1*, as well as amplification of *AURKA* and *MYCN* genes. Epigenetic alterations are postulated to be critical in the development of NEPC. Upregulation of EHZ2, a component of the polycomb repressive complex 2 (PRC2), is commonly found in NEPC, and its altered activity was shown to repress tumor suppressor genes, resulting in neuroendocrine transdifferentiation. Similarly, the PRC1 component CBX2, a chromodomain protein, was also found to be upregulated in NEPC tumors. Thus, upregulation of polycomb group (PcG) proteins leading to repression of PcG targets involving genes with a central role in neuroendocrine differentiation, are a hallmark of NEPC.

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Another route prostate cancer cells may employ to overcome enzalutamide treatment is through altered glucocorticoid receptor (GR) activity (Arora et al., 2013; Isikbay et al., 2014). GR is also a member of the SHR family and structurally similar to AR (Jimenez-Panizo et al., 2019). Expression of GR was found to be increased in CRPC patients that received enzalutamide (Arora et al., 2013). Importantly, the same study provided *in vitro* and *in vivo* data showing that prostate cancer cells with increased GR expression were able to proliferate in the presence of enzalutamide. Mechanistically, GR was shown to substitute AR function in prostate cancer cells in this context, with both receptors showing overlapping cistromes and transcriptomes (Arora et al., 2013; Sahu et al., 2013). Further study of GR upregulation in prostate cancer cells conferring enzalutamide-resistant growth as a result of overlapping receptor specificity and target gene activation by AR and GR, revealed the GR enhancer that controls GR expression in prostate cancer cells. AR and EZH2 were found to act in concert at this enhancer, repressing GR expression. Loss of both repressive AR binding and enhancer methylation was required for the increased expression of GR (Shah et al., 2017). Recently, in another study, GR activity as a mechanism of enzalutamide resistance was investigated in a panel of prostate cancer cell lines, showing an association between GR expression and enzalutamide response (Smith et al., 2020). These findings shed new light on GR function in prostate cancer and may have implications for CRPC patients who are often treated with glucocorticoids to suppress androgen biosynthesis. Clinical studies showed a significant PSA reduction in the majority of patients treated with glucocorticoids (Montgomery et al., 2014), illustrating clinical benefit as a result of glucocorticoid treatment. However, it was also shown that prostate cancer patients who were treated with enzalutamide, and received co-treatment with glucocorticoids, had worse survival compared to patients who did not receive glucocorticoids (Montgomery et al., 2014). Further research focusing on GR activity in prostate cancer and therapy response could improve the current application of regimens targeting AR and GR in the treatment of this disease.

Functional genetic screens

Loss-of-function functional genetic screens represent an effective approach for the identification of genes that have a central role in biological processes in various genetic and pharmacological backgrounds, including cancer and cancer therapy (Evers et al., 2016; Mulero-Sanchez et al., 2019; Mullenders & Bernards, 2009; Prahallad et al., 2012; Wang et al., 2017). This method harnesses the power of gene silencing techniques such as RNA interference (RNAi) and clustered regularly interspaced short palindromic repeat (CRISPR), applying them in large-scale gene perturbation experiments to screen for genes that drive phenotypes of interest (Evers et al., 2016). This approach not only

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augments in-depth understanding of complex biology, it also facilitates the discovery of genetic dependencies that may be exploited therapeutically. RNAi using short-hairpin RNA (shRNA) has largely been replaced by the CRISPR-Cas9 system in this setting, mainly due to the off-target activity and varying efficiency of shRNAs (Evers et al., 2016).
CRISPR technology adopts components of the bacterial immune system that are able to recognize and cleave DNA sequences in mammalian systems. CRISPR-mediated cleavage of the DNA results in a double strand break (DSB), for which the cells may utilize different repair mechanisms. One of these repair pathways is the error-prone non-homologous end joining (NHEJ) pathway, resulting in a loss-of-function mutation in the coding sequence of the gene and consequently loss of gene function (gene knockout).
The CRISPR-Cas9 system consists of two components that function in concert, namely the Cas9 endonuclease and a single guide RNA (sgRNA) molecule. The sgRNA associates with the Cas9 endonuclease to form a complex, after which the sgRNA interacts with its cognate sequence at the DNA allowing the Cas9 to perform its endonuclease activity there, cleaving the DNA. Thus, the sgRNA dictates sequence specificity of the complex, while the catalytic activity of Cas9 generates the DSB at the DNA (Makarova et al., 2006). Utilizing this mechanism, CRISPR screens may be used to interrogate a set of genes for their ability to control a binary response resulting in positive or negative selection in a certain background, such as a drug treatment (Evers et al., 2016). These binary responses translate to readouts such as cell survival vs cell death, senescence vs proliferation, activity vs inactivity of a reporter construct (Evers et al., 2016; Wang et al., 2018; Wang et al., 2017).
The collection of sgRNAs targeting a set of genes is known as a library, the composition of which can be selected depending on the aim of the study. These libraries can range from genome-wide sgRNA libraries, which target all the protein-coding genes in the genome (Sanjana et al., 2014), to smaller and focused sgRNA libraries that target a specific class of genes, such as kinases (Wang et al., 2018) or chromatin modifiers (Li et al., 2020). These CRISPR libraries are often used in a pooled format, meaning that a pooled population of cells is infected with the entire library, in a way that on average each cell is infected with a single sgRNA construct. Thus, the entire library is represented in a single pool of cells, with each cell typically containing ~1 sgRNA construct in the screen.
The ability of a specific CRISPR-mediated gene knockout to provoke a binary response, such as cell death vs survival, in a certain background is associated with enrichment or depletion of these cells and their sgRNAs, within the population. For example, if knockout of gene A causes cells to become resistant to a drug, then cells harboring the sgRNA targeting gene A will grow and expand in the presence of the drug, while the rest of the population will not. Conversely, if a CRISPR-mediated knockout of a gene causes cells to become more sensitive to a drug treatment, then these cells will be depleted and lost from the population. This positive or negative enrichment of sgRNAs within a population

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of cells under certain conditions can be quantified through massively parallel sequencing approaches, revealing candidate genes that are pivotal in generating the phenotype of interest (Fig. 3).

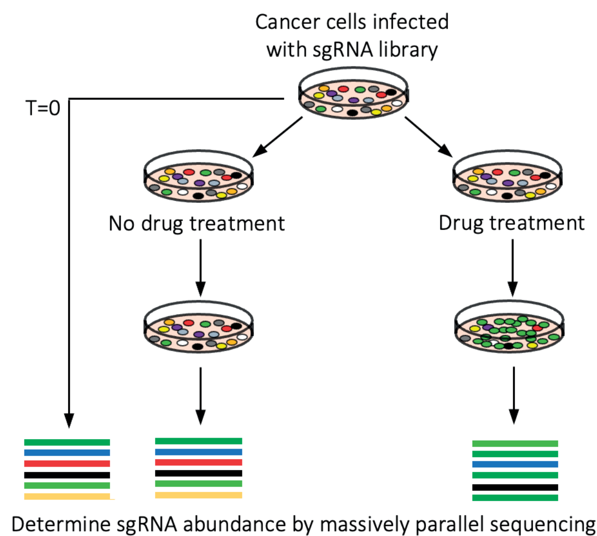

Figure 3: Setup of a pooled CRISPR-Cas9 screening approach to discover genes that modulate drug response. Cancer cells are infected with a CRISPR-Cas9 library containing sgRNAs targeting a set of genes. The cells are infected so that each cell contains on average ~1 sgRNA, making sure that the whole library is sufficiently represented. A sample is taken at the beginning of the screen (T=0) to determine the representation of the library the start. Cells are then cultured in the presence or absence of the drug. Depending on the interaction of the treatment with genes targeted by the library, the sgRNAs of genes may be enriched (here depicted in green), depleted or their relative abundance may remain stable. This is assessed at the end of the screen, when the sgRNA abundance/enrichment is determined by massively parallel sequencing, revealing candidate genes that control drug responses.

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Functional genetic screens have provided mechanistic insight into oncogenic signaling pathways and response to targeted therapies in cancer. For example, a kinome-centered shRNA screen focused on the response to BRAF inhibition in *BRAF(V600E)* colon cancer, revealed EGFR-mediated feedback activation as a determinant for poor clinical response of *BRAF V600E* colon cancer to vemurafenib monotherapy (Prahallad et al., 2012). This screen represents an éclatant example of a dropout screen, focused on investigating which shRNAs are depleted in *BRAF*-mutant colon cancer cells, as a result of vemurafenib treatment. The discovery that EGFR was the discriminating factor dictating BRAF inhibitor response in *BRAF*-mutant colon cancer cells, explained the discrepancy observed in the clinic where melanoma tumors with the same mutation show a significant vemurafenib response characterized by lack of EGFR feedback activation. These findings were translated to a clinical trial, showing improved survival in patients treated with inhibitors targeting BRAF, MEK and EGFR (Corcoran et al., 2018; Kopetz et al., 2019; Tabernero et al., 2021). Functional genetic screens can also be employed to study resistance, by investigating which sgRNAs enrich upon drug treatment. In this setup, sgRNAs are enriched as a consequence of the gene knockout mediated by the sgRNA, in combination with another variable, such as a drug treatment or a genetic trait (Sustic et al., 2018).

Altogether, CRISPR screens represent an unbiased approach to identify genetic determinants that dictate molecular processes controlling a variety of phenotypes, including cancer therapy response. This technique therefore facilitates drug target discovery and identification of predictive and prognostic biomarkers, ultimately leading to the improved stratification and treatment of cancer patients.

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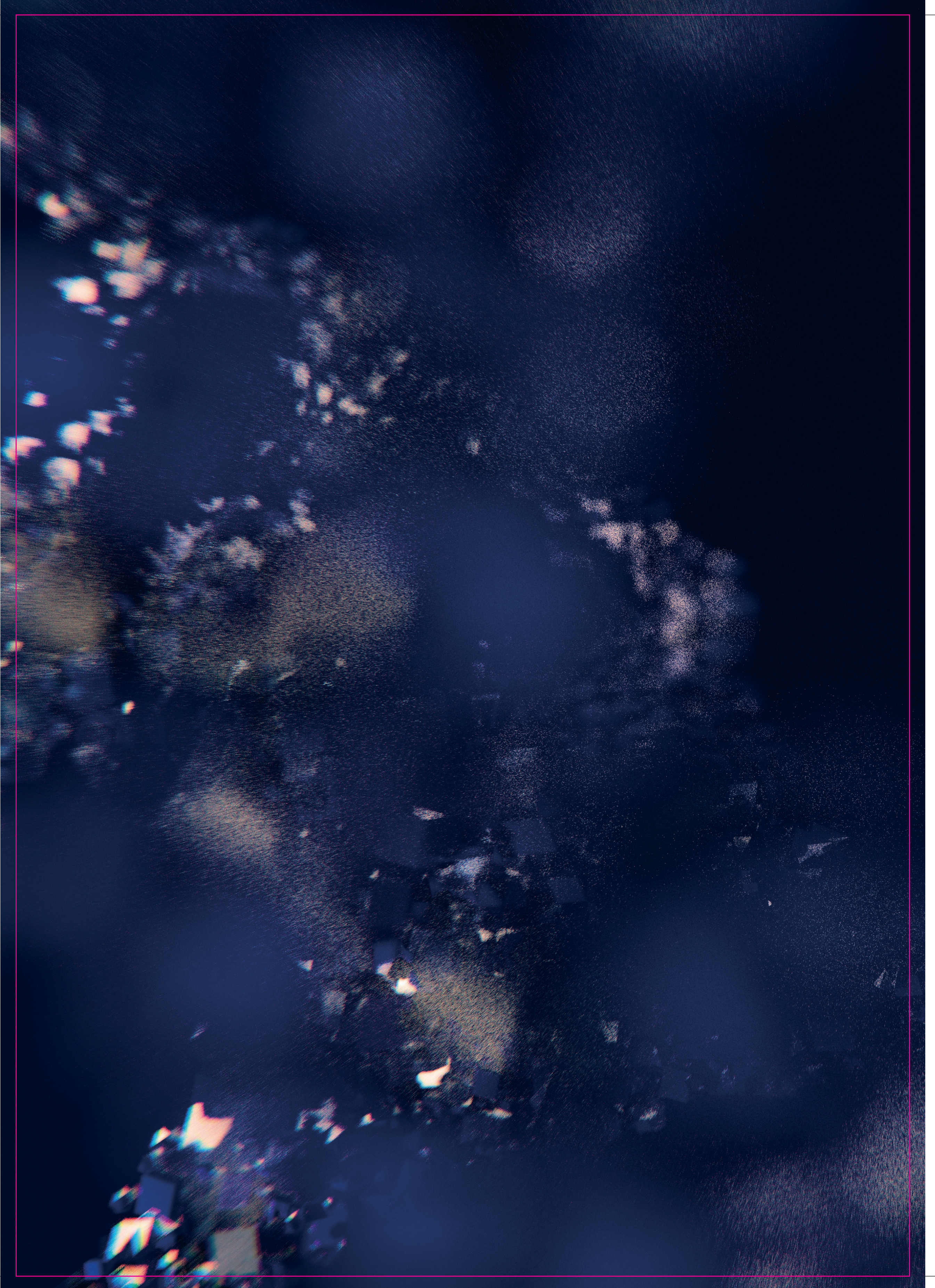

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Chapter 2

TLE3 loss confers AR inhibitor resistance by facilitating GR-mediated human prostate cancer cell growth

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Abstract

Androgen receptor (AR) inhibitors represent the mainstay of prostate cancer treatment. In a genome-wide CRISPR-Cas9 screen using LNCaP prostate cancer cells, loss of co-repressor *TLE3* conferred resistance to AR antagonists apalutamide and enzalutamide. Genes differentially expressed upon *TLE3* loss share AR as the top transcriptional regulator, and *TLE3* loss rescued the expression of a subset of androgen-responsive genes upon enzalutamide treatment. GR expression was strongly upregulated upon AR inhibition in a *TLE3*-negative background. This was consistent with binding of TLE3 and AR at the *GR* locus. Furthermore, GR binding was observed proximal to TLE3/AR-shared genes. GR inhibition resensitized *TLE3*^{KO} cells to enzalutamide. Analyses of patient samples revealed an association between TLE3 and GR levels that reflected our findings in LNCaP cells, of which the clinical relevance is yet to be determined. Together, our findings reveal a mechanistic link between TLE3 and GR-mediated resistance to AR inhibitors in human prostate cancer.

Introduction

Prostate cancer is the second most common cancer and the fifth leading cause of cancer-related death in men worldwide (Torre et al., 2012). Deregulated androgen receptor (AR) signaling is a major driver of prostate cancer (Taylor et al., 2010). Consequently, androgen deprivation therapy (ADT) is used to treat locally advanced and metastatic prostate cancer, achieving remission in most patients. However, despite castrate-levels of androgens in the serum, the disease inevitably progresses to a castration-resistant state (Perlmutter et al., 2007). AR signaling remains a pivotal driver in castration-resistant prostate cancer (CRPC), which is illustrated by the efficacy of AR-directed drugs such as abiraterone and enzalutamide. Unfortunately, patients develop resistance to these drugs and invariably succumb to the disease (Clegg et al., 2012; Beer et al., 2014; Chi et al., 2019).

Several resistance mechanisms to AR inhibitors have been proposed, including mutations in *AR* (Korpal et al., 2013; Joseph et al., 2013; Prekovic et al., 2016; Prekovic et al., 2018) and expression of splice variants (Li et al., 2013; Antonarakis et al., 2014; Culig et al., 2017). For example, the F877L missense mutation in *AR* was shown to confer resistance to enzalutamide and apalutamide (Korpal et al., 2013; Joseph et al., 2013; Balbas et al., 2013). Upregulation of the glucocorticoid receptor (GR, gene symbol *NR3C1*) was shown to be associated with clinical resistance to enzalutamide (Arora et al., 2013). Using the preclinical model LREX (LNCaP/AR Resistant to Enzalutamide Xenograft derived), it was shown that AR and GR have overlapping cistromes and transcrip-

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tomes, allowing GR to drive enzalutamide-resistant growth by regulating expression of a subset of AR target genes. (Arora et al., 2013; Shah et al., 2017). Significant overlap between AR and GR cistromes and transcription programs in prostate cancer cells was also described by others (Sahu et al., 2013). GR upregulation was found to occur through abrogation of the repressive function of AR and EZH2-mediated methylation of the *GR* enhancer (Shah et al., 2017). How exactly GR deregulation is mediated is incompletely understood. Combined, these studies have provided valuable insights into the molecular mechanisms underlying enzalutamide resistance in prostate cancer. However, to the best of our knowledge, a genome-scale approach aimed at identifying novel regulators of AR inhibitor sensitivity has hitherto not been reported. Loss-of-function genetic screens facilitate the unbiased identification of genes that have a central role in biological processes in various genetic or pharmacological backgrounds. Consequently, large-scale gene perturbation experiments are a powerful tool to identify novel drug targets and biomarkers of drug response (Mullenders et al., 2009). Using this technology, we aimed to discover genes not previously implicated in enzalutamide resistance. Through a genome-wide CRISPR-Cas9 screen we identified transducin-like enhancer of split 3 (*TLE3*) as a modulator of AR inhibitor sensitivity that, upon loss, confers resistance to enzalutamide in prostate cancer cells.

The well-conserved TLE protein family of transcriptional co-repressors is expressed in the nucleus of metazoans and regulate various biological processes including development, cell metabolism, growth and differentiation. At the chromatin, TLE protein family members maintain a silenced chromatin structure (Agarwal et al., 2015; Chen et al., 2000; Cinnamon et al., 2008). TLE3 is deregulated in various cancers including hormone-driven breast cancer (Jangal et al., 2014), colorectal cancer (Yang et al., 2016) and prostate cancer (Nakaya et al., 2007). Here, we report an unexpected role for TLE3 in regulating AR-mediated repression of the *GR* locus affecting AR inhibitor sensitivity in prostate cancer cells.

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Results

A genome-wide CRISPR-Cas9 resistance screen identifies TLE3 as a novel regulator of AR inhibitor sensitivity

The androgen-dependent prostate cancer cell line LNCaP is sensitive to AR inhibitors such as apalutamide (Figure 1-figure supplement 1A) and enzalutamide (Figure 1-figure supplement 1B), making it a model system well-suited for the unbiased discovery of novel regulators of AR inhibitor sensitivity in prostate cancer cells. LNCaP cells were infected with a lentiviral pool containing the genome-wide scale CRISPR Knock-Out (GeCKO) half-library A (Sanjana et al., 2014), targeting 19052 genes with 3 gRNAs per gene. Infected cells were cultured in the presence of vehicle or 2 μM of the AR inhibitor apalutamide for 6 weeks to allow selection of resistant cells. Subsequently, barcodes were recovered from the cells and submitted for massively parallel sequencing (Figure 1A and Figure 1-source data 1). DESeq2 (Love et al., 2014) analysis (Figure 1-source data 2) and MAGeCK (Li et al., 2014) analysis (Figure 1-source data 3) both identified *TLE3* as the top hit with all three gRNAs enriched in cells treated with apalutamide compared to untreated cells (Figure 1B and Figure 1-figure supplement 1C).

The screen was performed using apalutamide (Smith et al., 2018), which is a next-generation AR inhibitor structurally similar to enzalutamide. Subsequently, we validated the screen hit *TLE3* using both compounds. Abrogation of *TLE3* expression using independent single guide RNAs (sgRNAs), as well as short hairpin RNAs (shRNAs) targeting *TLE3*, conferred resistance to both enzalutamide and apalutamide in LNCaP cells in long-term growth assays (14 days) with drug concentrations up to 8 μM (Figure 1C-E and Figure 1-figure supplement 1D and 1E). Because enzalutamide is the current standard used in the clinic for the treatment of castration-resistant prostate cancer (CRPC), we used this drug for subsequent experiments. As prostate cancer is considered a heterogenous disease, for which only a few cell lines are available of which a subset is AR-driven, we next tested whether TLE3-mediated drug resistance could be confirmed in two other prostate cancer cell lines; CWR-R1 and LAPC4. As *TLE3* loss did not confer drug resistance in these two cell lines (Figure 1-figure supplement 1F-H), we conclude a context-dependency of this mode of resistance that is not commonly observed in all model systems.

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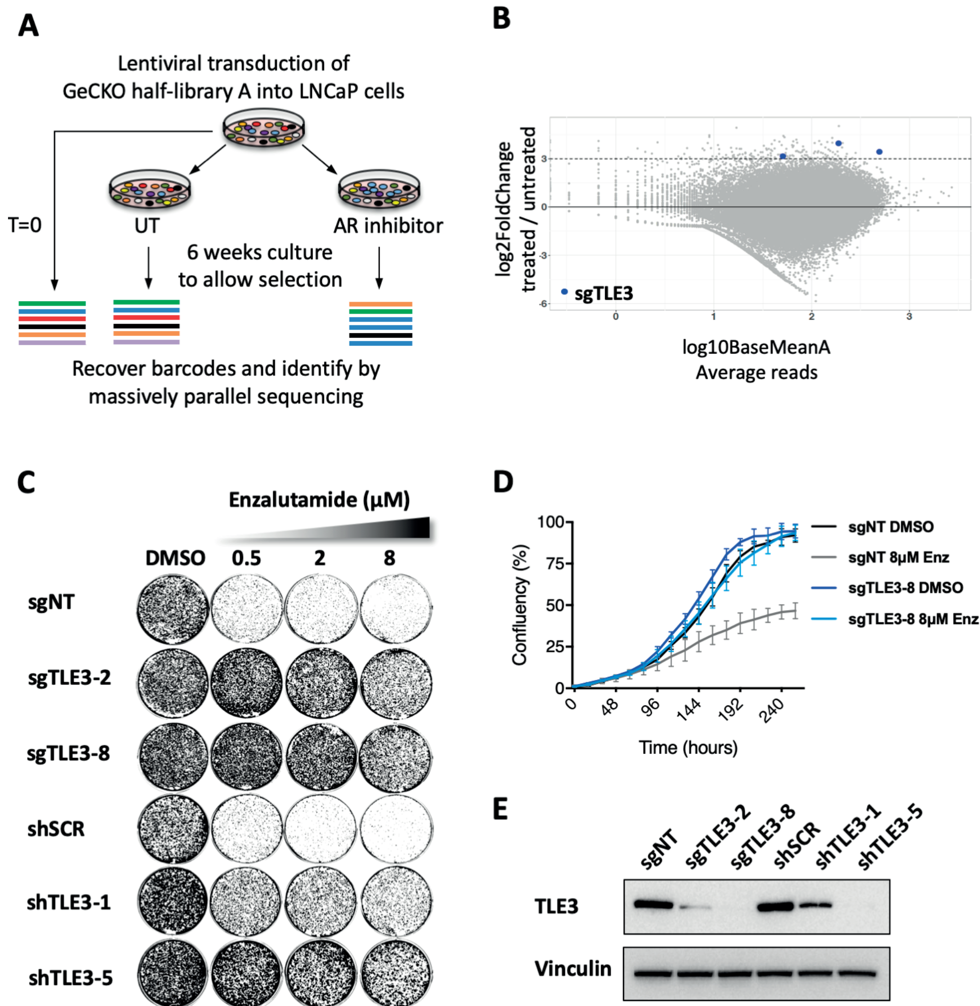

Figure 1: Genome-wide screen identifies TLE3 as a modulator of AR inhibitor sensitivity. (A) Overview of the genome-wide CRISPR-Cas9 resistance screen. (B) Representation of the relative abundance of the gRNA barcode sequences of the CRISPR-Cas9 resistance screen. The y-axis shows the enrichment (relative abundance of apalutamide treated/untreated) and the x-axis shows the average sequence reads of the untreated samples. (C) Long-term growth assays (14 days) showing the functional phenotype of LNCaP cells harboring TLE3 knockout or knockdown vectors, cultured in the presence of vehicle or enzalutamide. Cells harboring a non-targeting sgRNA (sgNT) or scrambled shRNA (shSCR) were used as a control. (D) Quantitative analysis of live cell proliferation in real-time for control cells and *TLE3*^{KO} cells in the absence or presence of enzalutamide. (E) Western blot showing TLE3 protein levels for control cells and *TLE3*^{KO} cells used in *C* and *D*.

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Loss of TLE3 leads to persistent expression of a subset of androgen-responsive genes in the presence of enzalutamide

TLE3 is also known to be a negative regulator of the Wnt pathway. However, analysis of active ß-catenin levels and expression of the *bona fide* Wnt target gene *AXIN2* in *TLE3*^{KO} cells treated with vehicle or enzalutamide revealed no changes compared to control cells (Figure 2-figure supplement 1A and B), indicating Wnt signaling is not altered in this context.

To investigate the transcriptional consequences of *TLE3* abrogation in LNCaP prostate cancer cells, the transcriptomes of control and *TLE3*^{KO} cells were compared (Figure 2-source data 1 and GSE130246). Because TLE3 is a transcription co-factor, we analyzed differentially expressed genes for transcription factor enrichment to explore which pathways could be involved in enzalutamide resistance conferred by *TLE3* loss. Enrichment analysis revealed AR as the top transcription factor associated with genes differentially expressed in control cells versus *TLE3*^{KO} cells in vehicle condition (Figure 2-figure supplement 1C). Genes differentially expressed in control cells versus *TLE3*^{KO} cells, cultured in the presence of enzalutamide, also shared AR as the top regulator (Figure 2A). An overview of the most differentially expressed genes in control cells versus *TLE3*^{KO} cells treated with enzalutamide is shown in Figure 2B. We validated expression for several of these genes both in the absence and presence of enzalutamide and found that loss of *TLE3* rescued expression of these genes in cells exposed to enzalutamide (Figure 2C and Figure 2-figure supplement 1D). We next asked the question whether general AR signaling is restored upon *TLE3* loss in enzalutamide-treated cells. To test this, we performed gene set enrichment analysis (GSEA) selectively focusing on AR-responsive genesets. Overall, AR signaling was maintained in the presence of enzalutamide in *TLE3*^{KO} cells but not in control cells, implying a rescue of AR signaling despite enzalutamide treatment (Figure 2D and 2E and Figure 2-figure supplement 1E).

Based on its role in the regulation of AR target genes and AR inhibitor resistance, we hypothesized that *TLE3* itself may be androgen-regulated. Indeed, western blot analysis showed that in wild-type (WT) cells, the expression of TLE3 is induced by enzalutamide (Figure 2-figure supplement 1F). Conversely, stimulation with the synthetic androgen R1881 led to a decrease in TLE3 protein levels (Figure 2-figure supplement 1F). Hormone manipulation led to similar changes in LAPC4 and CWR-R1 cells, although to a much lesser extent (Figure 2-figure supplement 1G). Analysis of publicly available ChIP-seq data (Stelloo et al., 2018) revealed binding of both TLE3 and AR at enhancer sites of the *TLE3* gene (Figure 2-figure supplement 1H) suggesting that these transcription factors regulate *TLE3* expression, indicating a feedback loop controlling *TLE3* transcription. This is supported by analysis of publicly available RNA-seq data (Massie et al. 2011) showing that *TLE3* mRNA levels are downregulated over time in LNCaP cells that are treated with R1881 (Figure 2-figure supplement 1I). Finally, we also investigated the effect of

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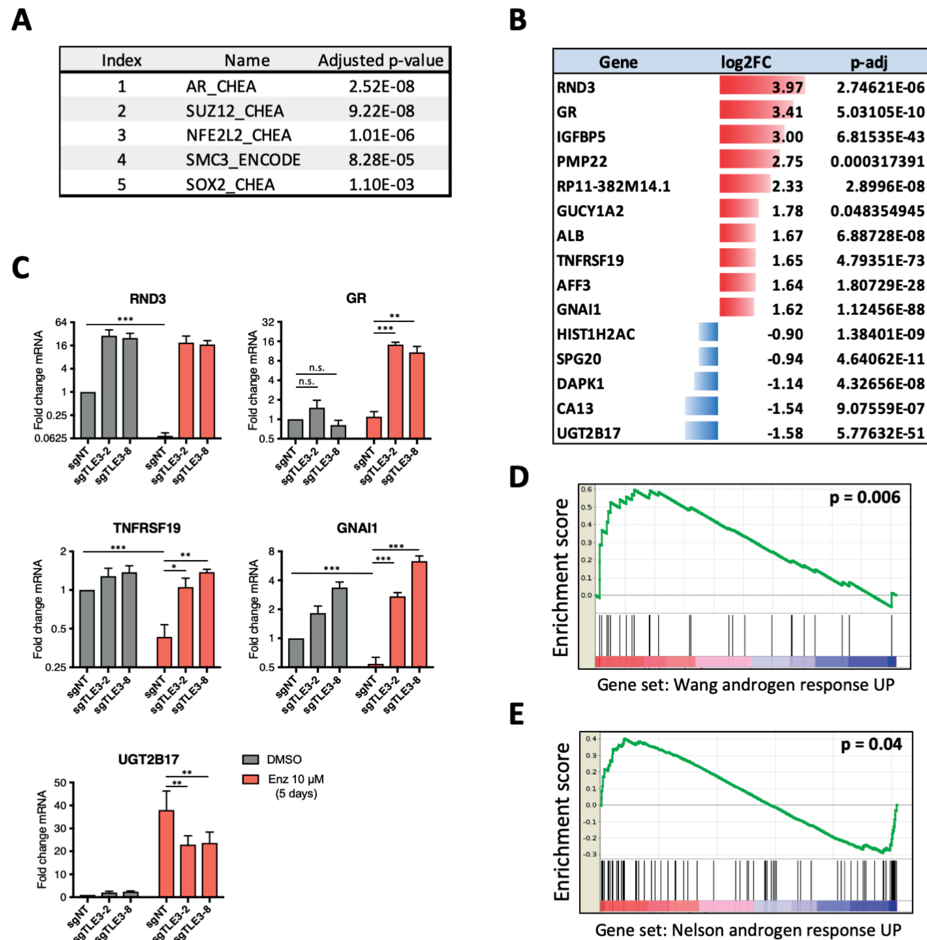
TLE3 loss on *AR* gene expression using qPCR and found no differential expression for *AR* mRNA between control and *TLE3*^{KO} cells (Figure 2-figure supplement 1J).



A

Index	Name	Adjusted p-value
1	AR_CHEA	2.52E-08
2	SUZ12_CHEA	9.22E-08
3	NFE2L2_CHEA	1.01E-06
4	SMC3_ENCODE	8.28E-05
5	SOX2_CHEA	1.10E-03

B

Gene	log2FC	p-adj
RND3	3.97	2.74621E-06
GR	3.41	5.03105E-10
IGFBP5	3.00	6.81535E-43
PMP22	2.75	0.000317391
RP11-382M14.1	2.33	2.8996E-08
GUCY1A2	1.78	0.048354945
ALB	1.67	6.88728E-08
TNFRSF19	1.65	4.79351E-73
AFF3	1.64	1.80729E-28
GNAI1	1.62	1.12456E-88
HIST1H2AC	-0.90	1.38401E-09
SPG20	-0.94	4.64062E-11
DAPK1	-1.14	4.32656E-08
CA13	-1.54	9.07559E-07
UGT2B17	-1.58	5.77632E-51

Figure 2: Transcriptomics analyses comparing control and *TLE3*^{KO} cells cultured in the presence of vehicle or 10 µM enzalutamide for 5 days. (A) Enrichment analysis for transcription factors associated with genes differentially expressed in enzalutamide-treated control cells compared to *TLE3*^{KO} cells. (B) Overview of the fold changes in gene expression of the most differentially expressed genes in control cells versus *TLE3*^{KO} cells treated with enzalutamide. (C) Validation (qPCR) of mRNA expression levels for several genes shown in *B*. Bars represent average data from at least three independent experiments ± SEM. P-values are indicated with ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ (two-tailed *t*-test). (D-E) GSEA for genes differentially expressed in control cells compared to *TLE3*^{KO} cells, treated with 10 µM enzalutamide using indicated gene sets.

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TLE3 localizes at AR binding sites proximal to genes differentially expressed in *TLE3*^{KO} cells compared to control cells

Gene expression profiling of *TLE3*^{KO} cells revealed persistent expression of androgen-responsive genes in the presence of enzalutamide. Recently, protein interactome profiling of AR revealed that TLE3 binds together with FOXA1 at androgen response elements (AREs) (Stelloo et al., 2018). We next analyzed publicly available ChIP-seq data (Stelloo et al., 2018) for the genome-wide binding profiles of AR, TLE3 and FOXA1 in LNCaP cells to explore the role of these transcription factors in the direct regulation of genes differentially expressed in control cells compared to *TLE3*^{KO} cells under enzalutamide treatment. Genes showing the strongest log2 fold-change expression in *TLE3*^{KO} compared to control cells were indeed bound by TLE3 (Figure 3-figure supplement 1A). In Figure 3A, the coverage profiles for TLE3 and AR are shown at the loci of two genes (*RND3* and *GNAI1*) whose expression was found to correlate with *TLE3*^{KO} and enzalutamide treatment (Figure 3A and Figure 2B and 2C). Genome-wide analysis of the binding patterns for AR, TLE3 and FOXA1 at the regulatory elements of differentially expressed genes extended our findings more broadly showing overlap for these proteins at these sites with markedly similar binding profiles observed for TLE3 and FOXA1 (Figure 3B). We found that co-binding of TLE3 and AR was enriched at loci of the differentially expressed geneset when compared to a random geneset (Figure 3C). Furthermore, significantly enriched sequence motifs at TLE3 binding sites of differentially expressed genes included members of the forkhead box transcription factor family (including FOXA1), AR, HOXB13 and the glucocorticoid receptor (GR) (Supplementary File 1). Since TLE3 acts as a repressor, the chromatin binding profiles for TLE3, FOXA1 and AR substantiate the expression data indicating that loss of *TLE3* alters expression of androgen-responsive genes towards an active-AR-like profile in spite of anti-hormonal treatment, thereby allowing continued growth when these cells are exposed to enzalutamide.

▶**Figure 3:** ChIP-seq analyses for transcription factor binding at differentially expressed genes in control cells compared to *TLE3*^{KO} cells cultured in the presence of 10 μM enzalutamide. (A) Coverage profiles for TLE3 and AR at the loci of two genes (*RND3* and *GNAI1*). (B) Heatmap of AR, TLE3 and FOXA1 (co-)binding at genes differentially expressed in *TLE3*^{KO} compared to control cells treated with enzalutamide are shown. The binding of AR, TLE3 and FOXA1 at these sites is shown for androgen-depleted or R1881-stimulated (4 hours) conditions in parental LNCaP cells. (C) ChIP-seq peak enrichment near the Transcription Start Sites (TSS) of differentially expressed (DE) genes and a random set of genes. The fraction of genes with a peak for TLE3, AR or both transcription factors at indicated distance from the TSS is shown for both genesets.

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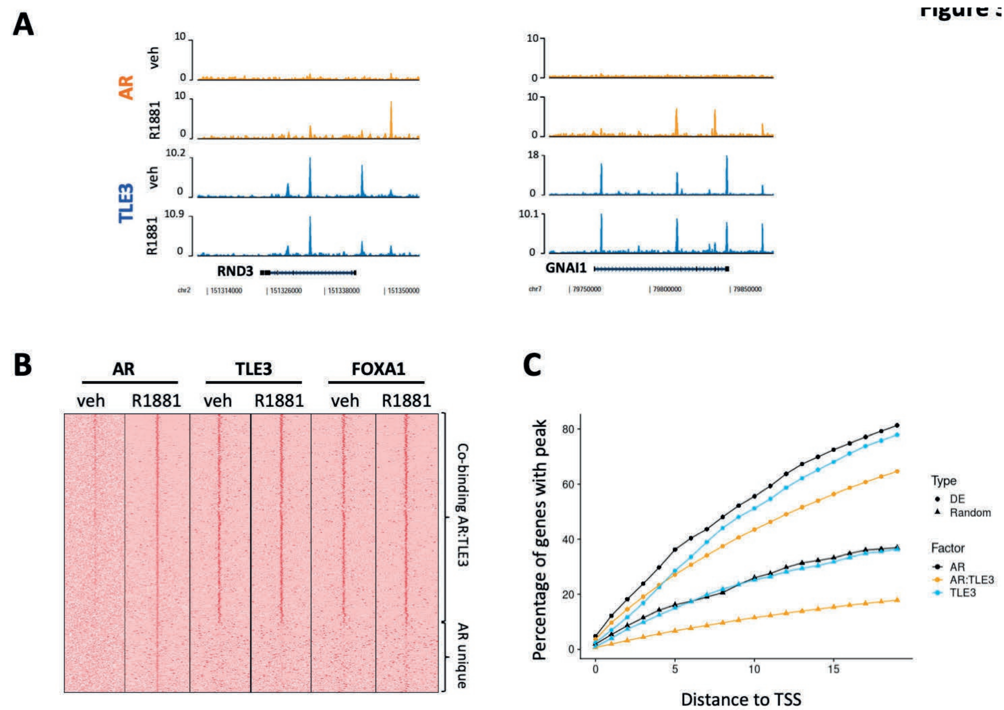

Enzalutamide resistance in *TLE3*^{KO} cells occurs through GR which is upregulated upon AR inhibition

Gene expression analysis revealed that *GR* was one of the most upregulated genes upon enzalutamide treatment in a *TLE3*-loss background (Figure 2B and 2C). Western blot analysis for GR confirmed this upregulation on protein level (Figure 4A). The binding of TLE3 and AR at the *GR* enhancer provides further evidence that both proteins play a role in the transcriptional repression of *GR* (Figure 4B). Moreover, TLE3 and AR binding at this region occurs at the same regulatory element described previously to be relevant in the regulation of *GR* in prostate cancer progression (Shah et al. 2017) (Figure 4-figure supplement 1A). The core GR and AR consensus sequences are highly similar (Figure 4-figure supplement 1B), and GR sequence motifs were enriched at genes differentially expressed in control versus *TLE3*^{KO} cells cultured with enzalutamide (Supplementary File 1). Interestingly, GR has been implicated in mediating resistance to AR inhibitors (Arora et al., 2014; Shah et al., 2017) so we decided to further investigate the link between TLE3 and GR in the context of antihormonal therapy. To assess whether GR can act as a key effector in *TLE3*^{KO} cells resulting in drug resistance, we performed inhibition experiments for this receptor in the context of enzalutamide treatment comparing control and *TLE3*^{KO} cells. Inhibition of GR using shRNAs in control and *TLE3*^{KO} cells resensitized *TLE3*^{KO} cells to enzalutamide (Figure 4C-E and Figure 4-figure supplement 1C). Inhibition of GR using the small molecule inhibitor mifepristone in conjunction with enzalutamide,

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reduced the proliferation of *TLE3*^{KO} when compared to single-drug treatments (Figure 4F). We next performed ChIP-qPCR to determine GR chromatin binding proximal to several of the most-differentially expressed genes in control versus *TLE3*^{KO} cells, in the presence of enzalutamide (listed in Figure 2B). This experiment showed binding of GR at these loci only in *TLE3*^{KO} cells treated with enzalutamide (Figure 4G). As TLE3 is known to recruit HDACs (Chen et al., 2000; Cinnamon et al., 2008), we also investigated histone acetylation at the GR locus and AR/TLE3 target gene RND3 and found that loss of TLE3 resulted in an upregulation of H3K27 acetylation at these enhancers (Figure 4-figure supplement 1D). Thus, abrogation of the repressive function mediated by both AR and TLE3 at the *GR* locus allows for increased expression of GR which, in turn, is able to confer enzalutamide resistance by substituting for AR in this context. Interestingly, we found overlap between several of the most-differentially expressed genes listed in Figure 2 (RND3, GNAI1, GR, UGT2B17 and PMP22) and GR-regulated genes described in a model for GR-mediated enzalutamide resistance as reported by others (Arora et al., 2013) (Figure 4-figure supplement 1E). These results are further supported by previous findings showing that AR and GR have overlapping transcriptomes and cistromes in the LNCaP-derived enzalutamide-resistant cell model LREX where GR was shown to confer enzalutamide resistance (Arora et al., 2014; Shah et al., 2017). Together, our data shows that loss of *TLE3* in conjunction with AR inhibition results in GR upregulation, leading to enzalutamide-resistance in LNCaP prostate cancer cells.

▶**Figure 4:** GR inhibition resensitizes *TLE3*^{KO} cells to enzalutamide treatment. (A) Western blot showing protein expression levels of TLE3 and GR in control and *TLE3*^{KO} cells cultured vehicle or enzalutamide (B) Coverage profiles for TLE3 and AR binding at the *GR* locus. (C) Long-term growth assay (14 days) showing the drug resistance phenotype in control and *TLE3*^{KO} cells with and without *GR* knockdown in the presence of vehicle or enzalutamide. (D) Western blot analysis for TLE3 protein levels in control and *TLE3*^{KO} cells shown in *C*, using GAPDH as a loading control. (E) mRNA levels for *GR* in control and *TLE3*^{KO} cells carrying shSCR or shGR constructs, shown in *C*. (F) Long-term growth assay (14 days) for cells harboring a control sgRNA or *TLE3*-targeting sgRNA cultured in the presence of vehicle, enzalutamide, mifepristone or the combination at indicated concentrations. (G) ChIP-qPCR showing GR occupancy at enhancers proximal to indicated genes. All samples were cultured in the presence of 10 μM enzalutamide with or without 1 μg/ml hydrocortisone (HC) as indicated.

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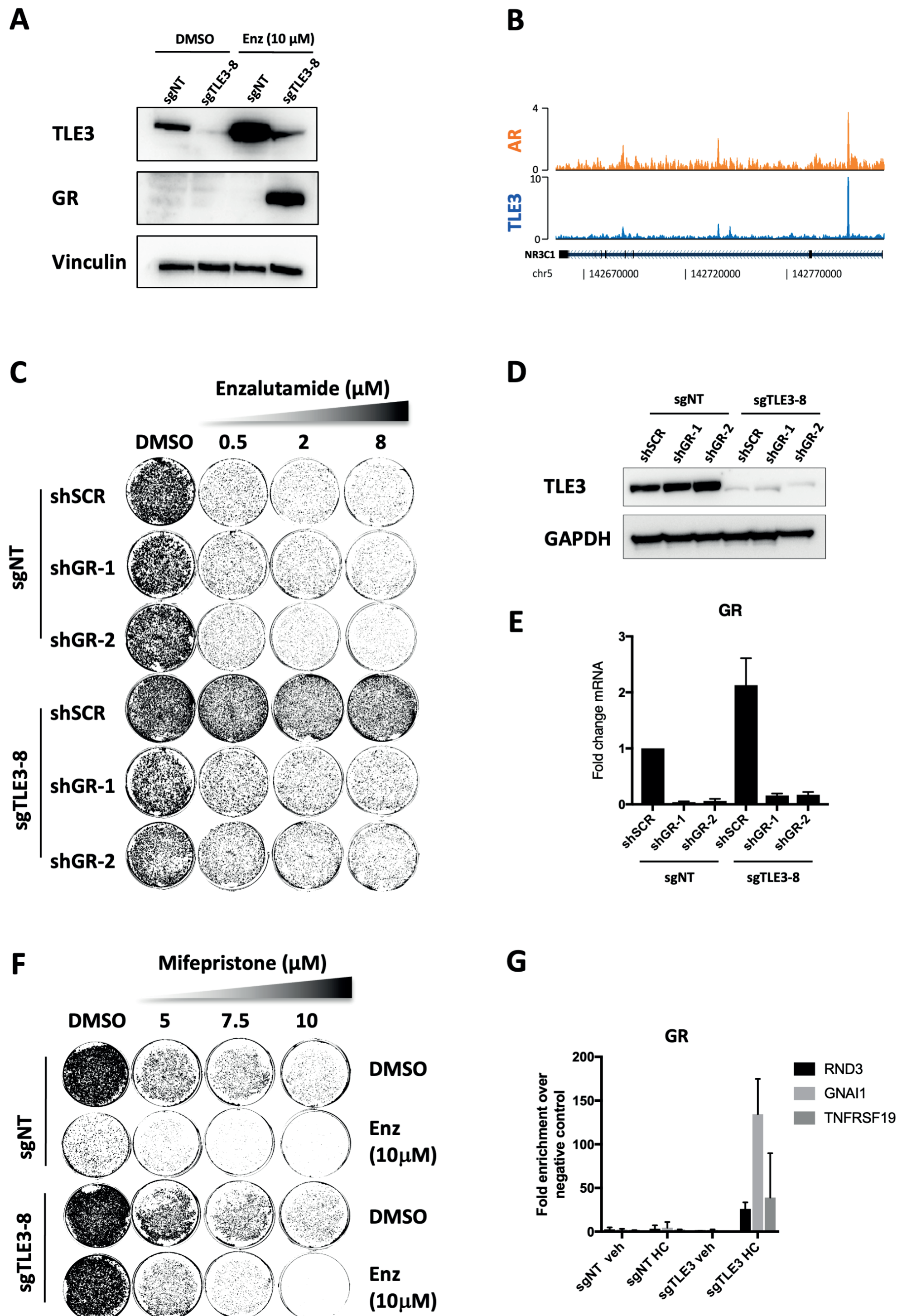

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TLE3 and GR expression are inversely correlated in prostate cancer patients and TLE3^{low}/GR^{high} expression is associated with poor response to antihormonal therapy

Analysis of two publicly available RNA-seq datasets (TCGA prostate and Abida et al., 2019) revealed an inverse correlation between TLE3 expression and GR expression in biopsy samples from prostate cancer patients with early-stage disease (Figure 5A) as well as advanced prostate cancer (Figure 5B).

We next investigated the effect of TLE3 expression levels on disease progression in prostate cancer patients. Analysis of the TCGA prostate cancer patient dataset filtered for patients who had undergone anti-hormonal therapy revealed a correlation between TLE3 expression and biochemical recurrence (p=0.033, n=65) (Figure 5C). These data show that TLE3 expression is a prognostic factor for prostate cancer patients treated with anti-hormonal therapy. As part of a clinical trial run in-house (PRESTO), matched tissue samples of metastatic sites were collected before treatment and after progression on enzalutamide treatment for four CRPC patients. These paired biopsies were analyzed by immunohistochemistry for expression of TLE3 and GR, to investigate whether expression of these proteins is altered upon selection pressure by enzalutamide. Two patients had a short PSA response to enzalutamide (<6 months), without radiological response. Tumor tissue of these patients showed moderate to high GR expression at baseline with weak or negative staining for TLE3 (Figure 5D, and Figure 5-figure supplement 1A). This was also observed in the post-treatment samples from these patients, in agreement with our hypothesis of low TLE3 and high GR in resistant tumors. Moreover, for one of these patients, the inverse association between TLE3 and GR became more pronounced upon enzalutamide treatment (Figure 5D). The third patient, having a more profound response (PSA response >12 months, radiological response), had weak staining for TLE3 and moderate staining for GR at baseline. In the post-treatment staining, TLE3 was low, whereas GR expression had increased (Figure 5D). The fourth patient had a protracted response to enzalutamide (>2 years) and showed low expression of both TLE3 and GR in pre- and post-treatment tissue (Figure 5-figure supplement 1A). In this patient, amplification of AR was observed upon treatment, potentially explaining resistance not related to TLE3 expression. Combined, these data show that TLE3 and GR are inversely correlated in prostate cancer patient samples and that low TLE3 and high GR expression were observed in several cases of enzalutamide resistance.

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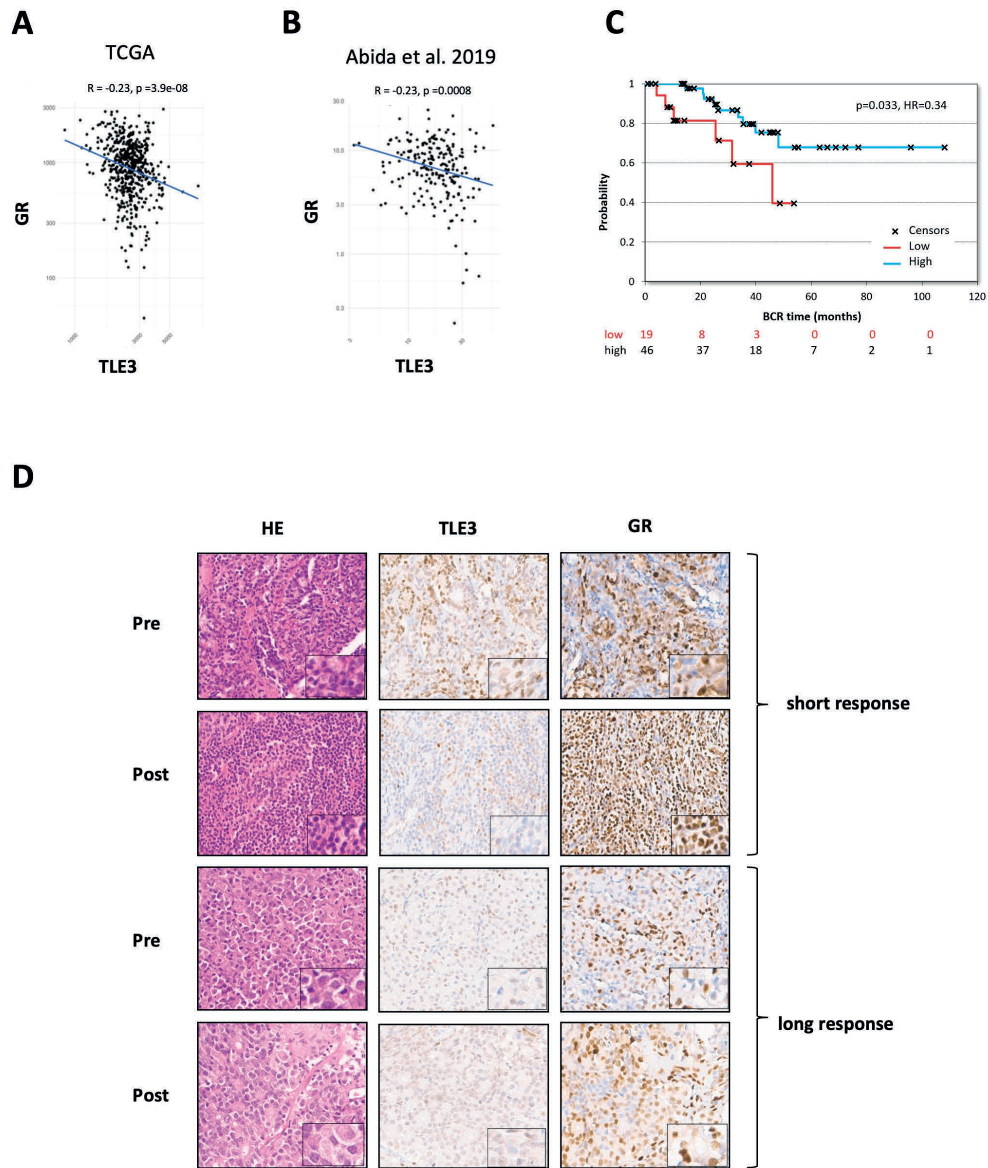

Figure 5: TLE3 and GR expression in tumors of prostate cancer patients. (A-B) RNA-seq analysis showing the correlation between TLE3 and GR expression in tumor samples from prostate cancer patients. (C) Kaplan-Meier curve showing the biochemical recurrence of prostate cancer patients from the TCGA dataset, only patients receiving anti-hormonal therapy were included (65 patients) using an optimal cut-off for high versus low TLE3 expression. (D) Immunohistochemistry for H&E, TLE3 and GR in tumor biopsy samples collected from two CRPC patients pre- and post-enzalutamide treatment.

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Discussion

The efficacy of the AR antagonists enzalutamide and apalutamide illustrates the importance of persistent signaling through the AR pathway in CRPC (Clegg et al., 2012; Beer et al., 2014). The transient nature of these drug responses underscores the relevance of improving therapeutic approaches and mechanistic understanding of drug resistance (Prekovic et al., 2018). Using a genome-wide CRISPR-Cas9 resistance screen we identified TLE3 as a novel regulator of AR inhibitor sensitivity that binds to and regulates the expression of androgen-responsive genes.
TLE3 was shown to co-localize with FOXA1 and AR at enhancer elements, which are selectively activated during prostate tumorigenesis (Stelloo et al., 2018), underscoring the importance of these transcription factors in this context. Our gene expression analyses show that loss of *TLE3* results in an active-AR-like profile despite anti-hormonal treatment. Our findings are in line with TLE3's known role as a transcriptional repressor (Agarwal et al., 2015; Chen G et al., 2000; Cinnamon et al., 2008) and the fact that TLE3 binds AR target genes. Similarly, TLE3 was described as a co-repressor in breast cancer cells, where it co-regulates the expression of a subset of ERα target genes (Jangal et al.,2014). The same study showed that the binding of TLE3 to the chromatin at ERα target genes was dependent on FOXA1 (Jangal et al., 2014).
Pathway reactivation or feedback activation of parallel signaling pathways are commonly described mechanisms found in drug-resistant tumors treated with targeted therapy (Prahallad et al., 2012; Pawar et al., 2018). In enzalutamide-resistant prostate tumors, upregulation of GR was described as a resistance mechanism where the receptor was able to substitute for AR and drive expression of a subset of target genes (Arora et al.,2013). In this study, the GR upregulation observed in the preclinical LREX model was not immediate in response to enzalutamide but required treatment with the drug for an extended amount of time for adaptation *in vitro* (Arora et al., 2013). This extended period of time needed for adaptation could suggest an acquired loss of TLE3 expression over time, resulting in deregulated GR expression. The work of Shah et al. (2017) showed that loss of the repressive signals of both AR binding and EZH2-mediated methylation of a tissue-specific enhancer at the GR locus lead to upregulation of GR and drug resistance in prostate cancer cells. TLE3 is a known transcriptional repressor and is able to bind the same GR enhancer (Figure 4B and Figure 4-figure supplement 1A). Our finding that TLE3 loss, in conjunction with AR inhibition, leads to GR upregulation provides deeper insight into the epigenetic regulation of the GR locus in prostate cancer cells and supports the previously undescribed role of TLE3 in conferring enzalutamide sensitivity via GR. GR occupancy at several TLE3/AR target genes provides further evidence for the role of GR in mediating enzalutamide resistance. Importantly, several of the most differentially expressed target genes in enzalutamide-treated control cells compared to $TLE3^{KO}$ cells

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(RND3, GNAI1, GR, UGT2B17 and PMP22) were previously described to be GR-regulated in a model of AR inhibitor resistance. Together, our results provide novel insights into the regulation of the *GR* locus in the context of AR inhibition in prostate cancer cells, implicating TLE3 as a regulator of GR-mediated enzalutamide resistance.

A limitation of our study is the fact that, of the *in vitro* models we tested, loss of *TLE3* conferred resistance to enzalutamide only in LNCaP cells and not in LAPC4 and CWR-R1. The availability of *in vitro* prostate cancer models is limited, and the heterogeneous nature of resistance mechanisms to antihormonal therapies in prostate cancer may explain why *TLE3* loss did not confer resistance to enzalutamide in LAPC4 and CWR-R1 cells. To study broader applicability, we investigated several clinical data-sets. Analysis of RNA expression in two prostate cancer patient cohorts, showed an inverse correlation between TLE3 and GR expression and worse prognosis of prostate cancer patients with low TLE3 expression treated with antihormonal therapy. Additionally, immunohistochemistry on GR and TLE3 of tumor tissue collected from CRPC patients pre- and post-enzalutamide treatment support our findings in LNCaP cells. Although these observations are in agreement with our hypothesis, the clinical implications of our findings are yet to be resolved and need to be determined in larger cohorts. Thus, our results warrant further investigation into the role of TLE3 and enzalutamide resistance in prostate cancer patients.

In summary, we have identified TLE3 loss as a novel resistance mechanism to AR-targeted therapeutics in prostate cancer cells. Based on previously reported work and the data in our study, we propose a model in which loss of TLE3 and AR function at the GR enhancer leads to upregulation of GR, which is able to substitute for AR, resulting in enzalutamide resistance (Figure 6). Our data, implicating TLE3 in the regulation of *GR* expression and drug resistance, complements increasing evidence describing the role of this receptor in bypassing AR blockade in prostate cancer cells.

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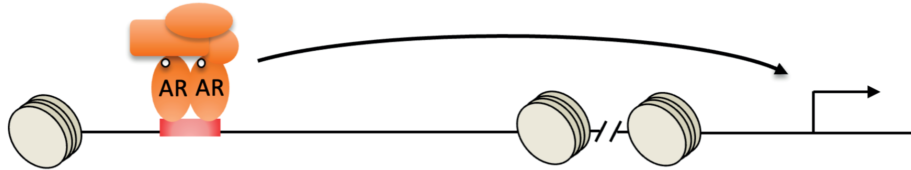
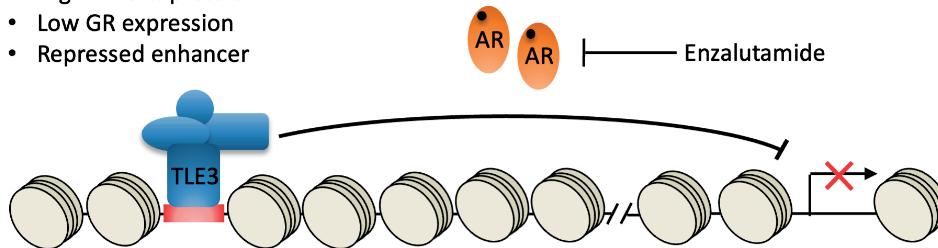
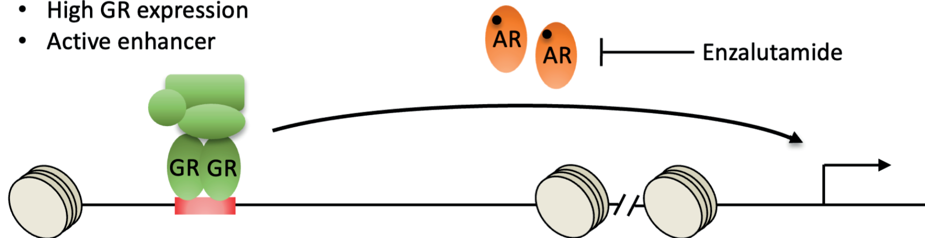
- Androgen-rich conditions
- Low TLE3 expression
- Low GR expression
- Active enhancer



- Enzalutamide treatment
- High TLE3 expression
- Low GR expression
- Repressed enhancer



- Enzalutamide treatment
- TLE3 loss of expression
- High GR expression
- Active enhancer



Hormone-responsive element (HRE)
Androgens
Enzalutamide

Figure 6: Model for GR-mediated enzalutamide resistance in *TLE3*^{KO} prostate cancer cells. In the presence of androgens, TLE3 expression is repressed and enhancers are active. AR regulates target gene expression, including repression of the *GR* locus (top panel). Upon enzalutamide treatment, TLE3 is upregulated and enhancers are inactive. TLE3 represses expression of AR target genes including *GR* (middle panel). Enzalutamide treatment in the context of *TLE3* loss leads to upregulation of GR which is able to substitute for AR at active enhancers, leading to drug resistance (bottom panel).

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Materials and methods

Cell culture and generation of knockout and knockdown cells

The human prostate cancer cell lines were maintained in RPMI (LNCaP, CWR-R1, 22rv1) or IMDM (LAPC4). HEK293T cells were cultured in DMEM. Medium was supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in 5% CO_2. All cell lines were STR profiled. Control and *TLE3*^{KO} cells were created by infecting target cells with lentiviral particles containing LentiCRISPR v2.0 harboring non-targeting or *TLE3*-targeting gRNAs, which were cloned in using Gibson Assembly (NEB cat#: E2611S) utilizing BsmBI restriction sites. For gRNA and shRNA sequences see Supplementary File 2: Key Resources Table. HEK293 were co-transfected with lentiviral CRISPR, or in-house shRNA constructs, using PEI. Target cells were seeded 1 day prior to infection. Lentiviral supernatant was added to the medium along with 5 μg/ml polybrene. Infected cells were selected with 2 μg/ml puromycin.

CRISPR-Cas9 resistance screen

LNCaP cells were infected with lentiviral particles containing GeCKO half-library A at low M.O.I. (~0.2) for single viral integration, at a ~150-fold coverage, and cultured in the presence of vehicle or 2 μM apalutamide for 6 weeks. Barcodes were recovered and sequenced as described (Brunen et al., 2018). DESeq2 (Love et al., 2014) analysis was performed using a paired design. The treated samples were compared with the untreated samples. A sgRNA was considered to be a hit, if the log2FC >= 3 and the FDR <= 0.1. TLE3 was the only gene for which all three sgRNAs were a hit. The MAGeCK (Li et al., 2014) analysis was done using the default settings, which produced TLE3 as top hit with a FDR of 0.002.

Proliferation assays

Colony formation assays were performed as previously described (Brunen et al., 2018). Used seeding densities were 20,000 (LNCaP, LAPC4) or 10,000 (22rv1, CWR-R1) cells/well in 6-well plates. After 12-14 days of growth in presence of the drugs as indicated, when control cells reached confluence, all cells were fixed in 2% formaldehyde and stained with 0.1% crystal violet.

Live cell proliferation was monitored in real-time using the Incucyte ZOOM (11 days). Cells were seeded in a 384-well plate at 600 cells/well and drugs were added as indicated. R1881, apalutamide, enzalutamide, mifepristone (Medkoo Biosciences) were dissolved in DMSO and stored at -20^{0}C.

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Protein lysate preparation and western blot analysis

Typically, LNCaP cells were plated at density of 200,000 cells in 6-well plates and cultured in the presence of enzalutamide for 5 days before harvesting. Samples were prepared and western blot was performed as described previously (Brunen et al., 2018), using primary antibodies directed against TLE3 (Santa Cruz Biotechnology, #sc-514798, 1:250), Vinculin (Sigma-Aldrich, #V4139, 1:1000), and GAPDH (Cell Signaling Technology, #5174S, 1:10000). Secondary antibodies were obtained from Bio-Rad laboratories.

RNA-seq

RNA-seq data was generated by seeding 500,000 LNCaP control or *TLE3*^{KO} cells in 10 cm dishes in the presence of 10 μM enzalutamide or vehicle for 5 days, followed by RNA isolation using the ISOLATE II RNA mini kit (Bioline). RNA was then submitted for Illumina sequencing (HiSeq 2500). The differential expression was based on the ratio of normalized read counts (FPKM, after library size correction). An absolute fold-change threshold of 2 was used. Genes with a coverage <50 in both conditions were excluded from the analyses to prevent spurious results. Data were further analyzed using Enrichr (Chen et al., 2013) and javaGSEA desktop application (http://software.broadinstitute.org/gsea). Data was uploaded to GEO (GSE130246).

Quantitative RT-PCR

LNCaP cells were plated at density of 200,000 cells in 6-well plates and cultured in the presence of enzalutamide for 5 days before harvesting. Total mRNA isolation, cDNA synthesis and qPCR analysis were performed as described elsewhere (Brunen et al., 2018). An overview of the used primers is listed in Supplementary File 2: Key Resources Table.

ChIP-seq and ChIP-qPCR

The ChIP-seq data from Figure 3 was sourced from Stelloo et al. (2018), GSEA94682. The sequencing (bam) files and the peaks called by Peaks were called using DFilter (Kumar V et al. Nat Biotechnol 2013;31(7):615-22) and MACS peak caller version 1.4 (Zhang Y et al. Genome Biol 2008;9(9):R137). The ChIP peaks were sorted by intensity. For each set of differentially expressed genes, the genomic locations were intersected with the peaks called, padding with 20 Kb for genes and 5 Kb for peaks prior to intersecting. Seqminer (Ye et al., 2011) was used to obtain the coverage data at the intersecting regions, and to generate the heatmaps. Coverage profile snapshots were made using Easeq (Lerdrup et al., 2016).

ChIP-qPCR data was generated according to the protocol described by Singh et al., 2018. Cells were plated at ~30% confluency in 15 cm dishes and cultured in the presence of 10 μM enzalutamide for 5 days. In case of hydrocortisone stimulation, hydrocortisone was added 2 hours prior to harvesting of the cells. The antibodies that were used were: 7,5 μl of

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anti-GR (CST, #12041) and 5μg of H3K27ac (Active Motif, 39133). Regions for qPCR were selected based on AR ChIP-seq data in Figure 3, choosing the peaks closest the target gene. For an overview of the primers see Supplementary File 2: Key Resources Table.

Immunohistochemistry

Immunohistochemistry of the FFPE tumor samples was performed on a BenchMark Ultra autostainer (TLE3) or Discovery Ultra autostainer (Glucocorticoid Receptor). Briefly, paraffin sections were cut at 3 μm, heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 64 minutes at 95°C.Glucocorticoid Receptor clone D6H2L (Cell Signaling) was detected using 1/600 dilution, 1 hour at 37°C and TLE3 using clone CL3573 (1/250 dilution, 1 hour at RT). Bound TLE3 was detected using the OptiView DAB Detection Kit (Ventana Medical Systems). Glucocorticoid Receptor bound antibody was visualized using Anti-Rabbit HQ (Ventana Medical systems) for 12 minutes at 37°C, Anti-HQ HRP (Ventana Medical systems) for 12 minutes at 37°C, followed by ChromoMap DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems).

Acknowledgements

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Competing interests

Wilbert Zwart: Reviewing editor, eLife. The other authors declare that no competing interests exist.

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Supplementary Figures

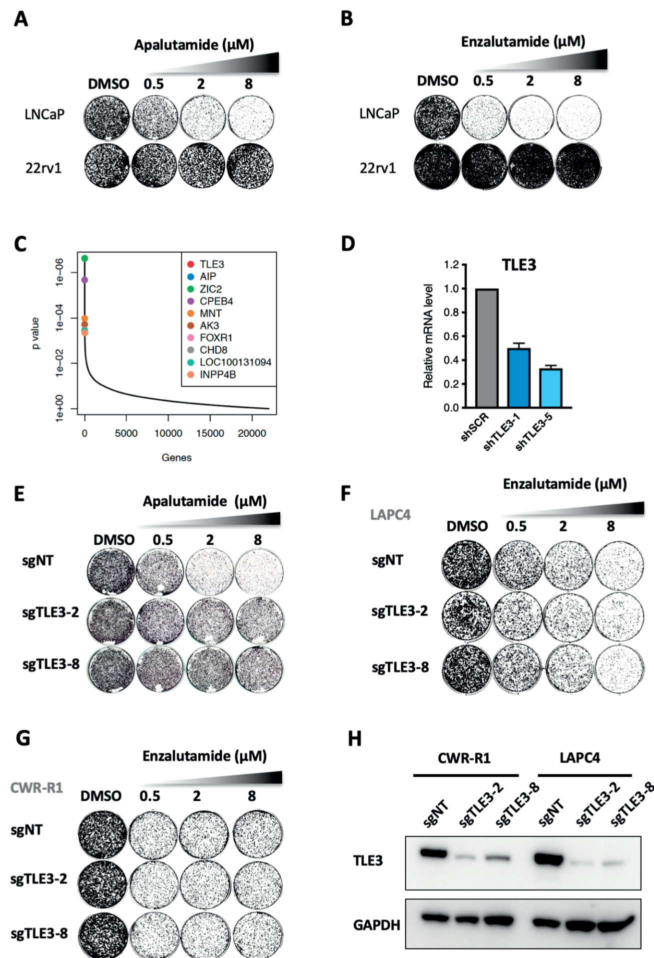

Figure 1-figure supplement 1: (A-B) Long-term growth assay of LNCaP cells treated with apalutamide or enzalutamide for 14 days. 22rv1 cells were used as control. (C) MAGeCK analysis showing hits obtained from the CRISPR-Cas9 resistance screen. (D) *TLE3* mRNA expression levels in LNCaP cells harboring shRNAs targeting *TLE3*. Cells with scrambled shRNA (shSCR) were used as a control. (E) Long-term growth assays showing the functional phenotype for control and *TLE3*^{KO} LNCaP cells cultured in the presence of vehicle or apalutamide at indicated concentrations for 14 days. (F-G) Long-term growth assays showing the functional phenotypes for indicated cell lines carrying control or *TLE3*-targeting gRNAs cells, cultured in the presence of vehicle or enzalutamide at indicated concentrations. (H) Western blot analysis showing TLE3 expression levels for the cells shown in *F* and *G*. GAPDH was used as a loading control.

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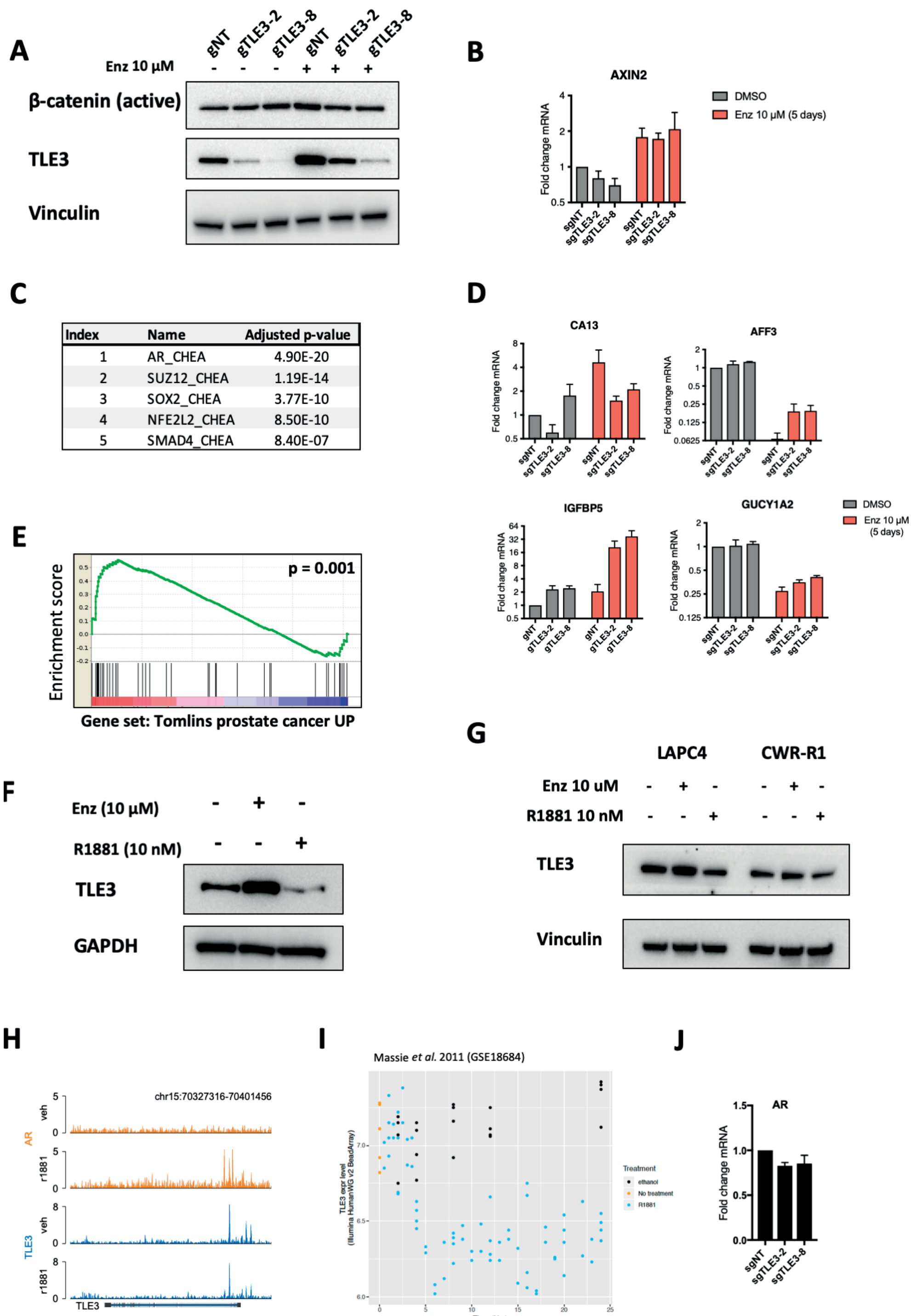

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◀**Figure 2-figure supplement 1**: (A) Western blot results showing expression levels of active ß-catenin, TLE3 and Vinculin (loading control) in indicated cell lines cultured with and without enzalutamide. (B) Expression levels of *AXIN2* as determined by qPCR in control and *TLE3*^{KO} cells cultured with and without enzalutamide. (C) Top five transcription factors associated with genes differentially expressed in control versus *TLE3*^{KO} cells in the untreated condition. (D) Validation of several genes most differentially expressed in *TLE3*^{KO} cells compared to control cells cultured in the presence of enzalutamide. (E) GSEA for genes differentially expressed in control versus *TLE3*^{KO} cells treated with enzalutamide, using the indicated geneset. (F) Western blot analysis for TLE3 expression levels in LNCaP cells cultured as indicated for 5 days. GAPDH was used as a loading control. (G) Western blot showing TLE3 protein expression levels in LAPC4 and CWR-R1 cells cultured with indicated drugs for 5 days. (H) Snapshot of coverage profiles for TLE3 and AR binding at the *TLE3* locus under indicated conditions. (I) RNA-seq data showing TLE3 mRNA levels of LNCaP cells treated with vehicle or R1881 for 24 hours (time course). (J) RT-qPCR analysis showing AR expression in untreated control and *TLE3*^{KO} cells.

A

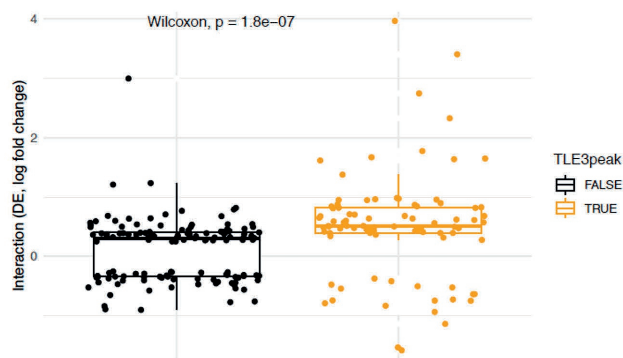

Figure 3-figure supplement 1: (A) Boxplot showing the Log2 fold change expression of genes associated with *TLE3*^{KO} and enzalutamide treatment. Genes have been grouped based on TLE3 binding status at the loci of these genes; no TLE3 binding (black) and TLE3 binding (orange).

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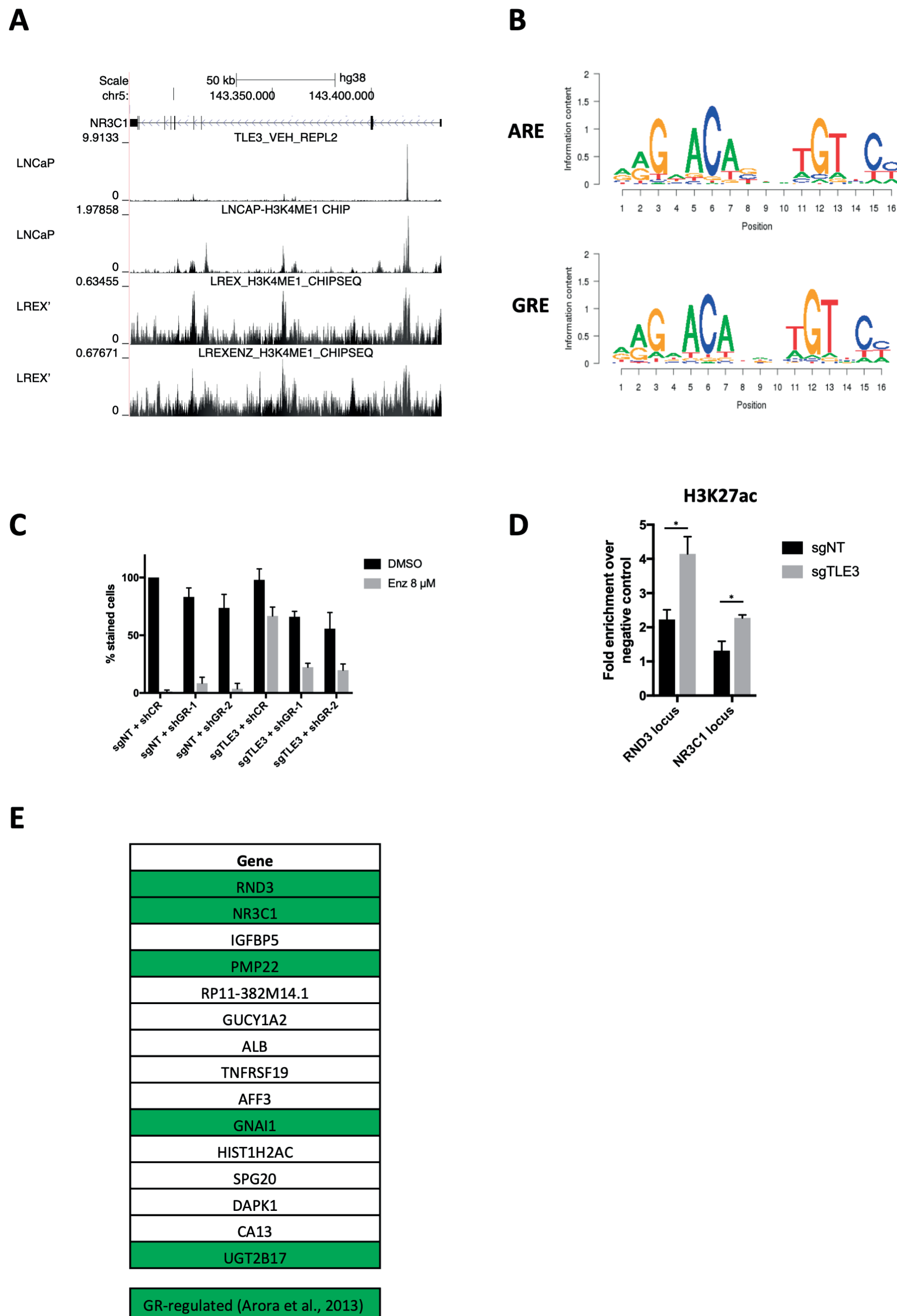

Gene
RND3
NR3C1
IGFBP5
PMP22
RP11-382M14.1
GUCY1A2
ALB
TNFRSF19
AFF3
GNAI1
HIST1H2AC
SPG20
DAPK1
CA13
UGT2B17

GR-regulated (Arora et al., 2013)

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◀**Figure 4-figure supplement 1**:
(A) Coverage profiles for TLE3 or H3K4me1 at the *GR* locus in LNCaP and LREX' cells. (B) Core consensus sequences for AR and GR. (C) Quantification of long-term colony formation assays showing the functional phenotype of control and *TLE3*^{KO}, with or without *GR* knockdown, in cells treated with vehicle or enzalutamide. (D) ChIP-qPCR for H3K27 acetylation in control and *TLE3*^{KO} cells at indicated loci. (E) Overview of the genes listed in Fig. 2B with GR-regulated genes (as shown by Arora et al., 2013) highlighted in green.

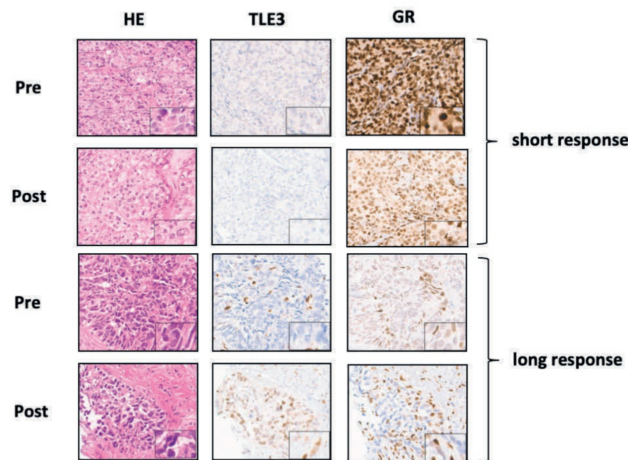

Figure 5-figure supplement 1: (A) Immunohistochemistry for H&E, TLE3 and GR in tumor biopsy samples collected from two CRPC patients pre- and post-enzalutamide treatment.

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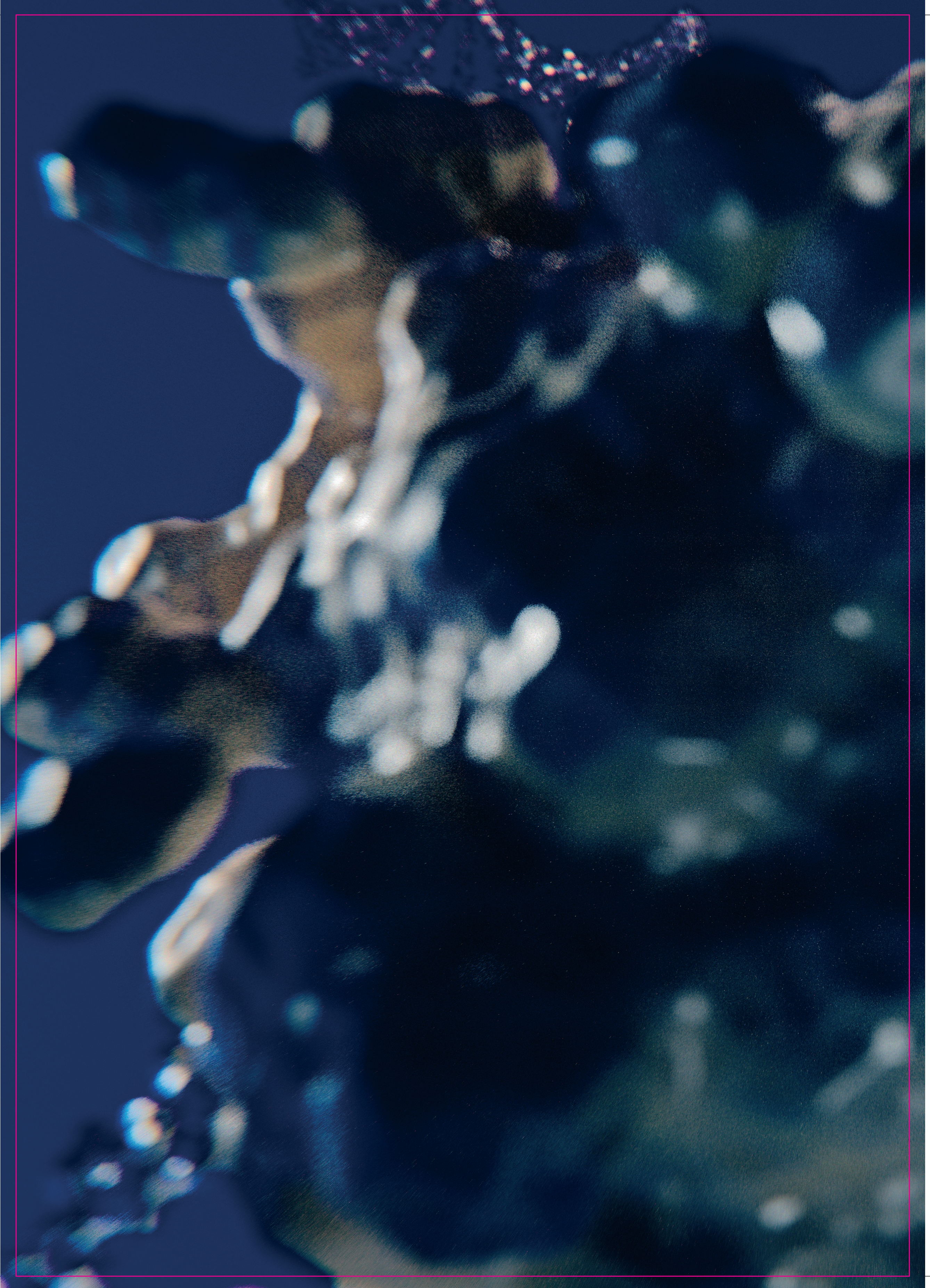

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Chapter 3

A kinome-centered CRISPR-Cas9 screen identifies activated BRAF to modulate enzalutamide resistance with potential therapeutic implications in *BRAF*-mutated prostate cancer

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Abstract

Resistance to drugs targeting the androgen receptor (AR) signaling axis remains an important challenge in the treatment of prostate cancer patients. Activation of alternative growth pathways is one mechanism used by cancer cells to proliferate despite treatment, conferring drug resistance. Through a kinome-centered CRISPR-Cas9 screen in CWR-R1 prostate cancer cells, we identified activated BRAF signaling as a determinant for enzalutamide resistance. Combined pharmaceutical targeting of AR and MAPK signaling resulted in strong synergistic inhibition of cell proliferation. The association between BRAF activation and enzalutamide resistance was confirmed in two metastatic prostate cancer patients harboring activating mutations in the *BRAF* gene, as both patients were unresponsive to enzalutamide. Our findings suggest that co-targeting of the MAPK and AR pathways may be effective in patients with an activated MAPK pathway, particularly in patients harboring oncogenic *BRAF* mutations. These results warrant further investigation of the response to AR inhibitors in *BRAF*-mutated prostate tumors in clinical settings.

Introduction

Prostate cancer is the second most common cancer diagnosed in men, accounting for over 350,000 cancer-related deaths worldwide each year[1]. The androgen receptor (AR) pathway is a key driver in prostate tumorigenesis, regulating genes that drive prostate cancer cell proliferation[2]. In recent years, new compounds have been introduced clinically that target the AR signaling axis resulting in tumor regression. These include drugs such as abiraterone, which blocks biosynthesis of androgen precursor molecules, and enzalutamide, which functions through antagonistic binding of AR. Even though these AR-directed drugs have shown to be clinically effective[3,4], evasion of AR blockade through adaptation inevitably leads to disease progression and eventually death[5,6,7]. Acquired resistance to enzalutamide has been the focus of intense research, and several mechanisms have been described. These resistance mechanisms include activation of other signaling pathways such as the PI3K pathway[8], NF-κB signaling [9] and glucocorticoid receptor (GR) overexpression[10,11].

Primary resistance is commonly defined by unresponsiveness to treatment, characterized by clinical progression within the first 3 months after commencing systemic therapy[5]. Primary resistance to enzalutamide, even though relatively under-examined, occurs in about 10% to 20% of prostate cancer patients[3,12]. A better mechanistic understanding of primary resistance will allow for better patient stratification and improved therapeutic avenues.

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Functional genetic screens using CRISPR-Cas9 are a powerful tool for the unbiased identification of genes that have a central role in a wide range of biological processes in various genetic and pharmacological backgrounds, including cancer[11,13,14]. For example, through a genome-wide CRISPR-Cas9 screen, TLE3 was identified as a novel modulator of enzalutamide sensitivity which, together with AR, regulates GR expression and drug response in prostate cancer[11]. Using a similar approach, we set out to identify kinases whose inhibition could potentiate enzalutamide efficacy in prostate cancer cells, with the aim to discover biomarkers for resistance and potential drug combinations that are able to overcome enzalutamide resistance. We found that inhibition of BRAF, or downstream MAPK components MEK and ERK, enhanced enzalutamide sensitivity in prostate cancer cells harboring a mutation in the activating kinase domain of the *BRAF* gene. Our findings suggest therapeutic potential for co-inhibition of the MAPK and AR pathways in *BRAF*-mutated prostate cancers.

Results

A kinome-centered dropout screen identifies BRAF as a modulator of enzalutamide sensitivity

The AR inhibitor enzalutamide is successfully used for the treatment of prostate cancer. However, primary resistance is observed in a significant proportion of patients[3,12], illustrating the need for improved therapeutic approaches for this subset of patients. To address this unmet clinical need, we performed a kinome-centered CRISPR-Cas9 screen to identify kinases whose inhibition synergize with AR inhibition in cells that show a poor response to enzalutamide. The cell line CWR-R1 is a prostate cancer cell line that shows moderate sensitivity to the AR inhibitor enzalutamide, as compared to the sensitive LNCaP cells and resistant 22rv1 cells (Fig. 1A-B). This moderate sensitivity makes the CWR-R1 cell line a suitable model system to screen for kinases whose inhibition may synergize with enzalutamide to enhance anti-tumor effects *in vitro*.

CWR-R1 cells were infected with the NKI Human Kinome CRISPR pooled sgRNA library targeting 578 human kinases. Infected cells were seeded at low density and treated with 10 µM enzalutamide or vehicle for 2 weeks to allow selection. The single gRNA (sgRNA) cassettes were recovered from the genomic DNA by PCR and their relative abundance was determined through massively parallel sequencing (Fig. 1C). The sgRNA abundance of the enzalutamide-treated and vehicle-treated populations were compared, and depleted sgRNAs were identified using DESeq2[15] and MAGeCK[16] analyses (Supplemental Table S1 and S2). We found that all 10 sgRNAs targeting *BRAF* were under-represented in enzalutamide-treated cells when compared to vehicle-treated cells (Fig. 1D).

We validated the results of the CRISPR-Cas9 screen using sgRNAs targeting *BRAF* in

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CWR-R1 cells, using polyclonal knockout cell populations. Knockout of *BRAF* using two independent sgRNAs (sg*BRAF*-9 and sg*BRAF*10) yielded viable cells with growth kinetics mirroring those of control cells harboring a non-targeting sgRNA (sgNT) (Fig. 1E and 1F). CRISPR-mediated loss of BRAF protein expression in *BRAF*^{KO} cells was confirmed by western blot (Fig. 1G). Importantly, *BRAF*^{KO} cells showed increased sensitivity to enzalutamide in long-term growth assays when compared to control cells (Fig. 1E and 1F), concordant with the results from the screen. In contrast to our findings in CWR-R1 cells, knockout of *BRAF* using CRISPR-Cas9 in LNCaP cells did not result in increased sensitivity to enzalutamide (Supplemental Fig. S1A-C).

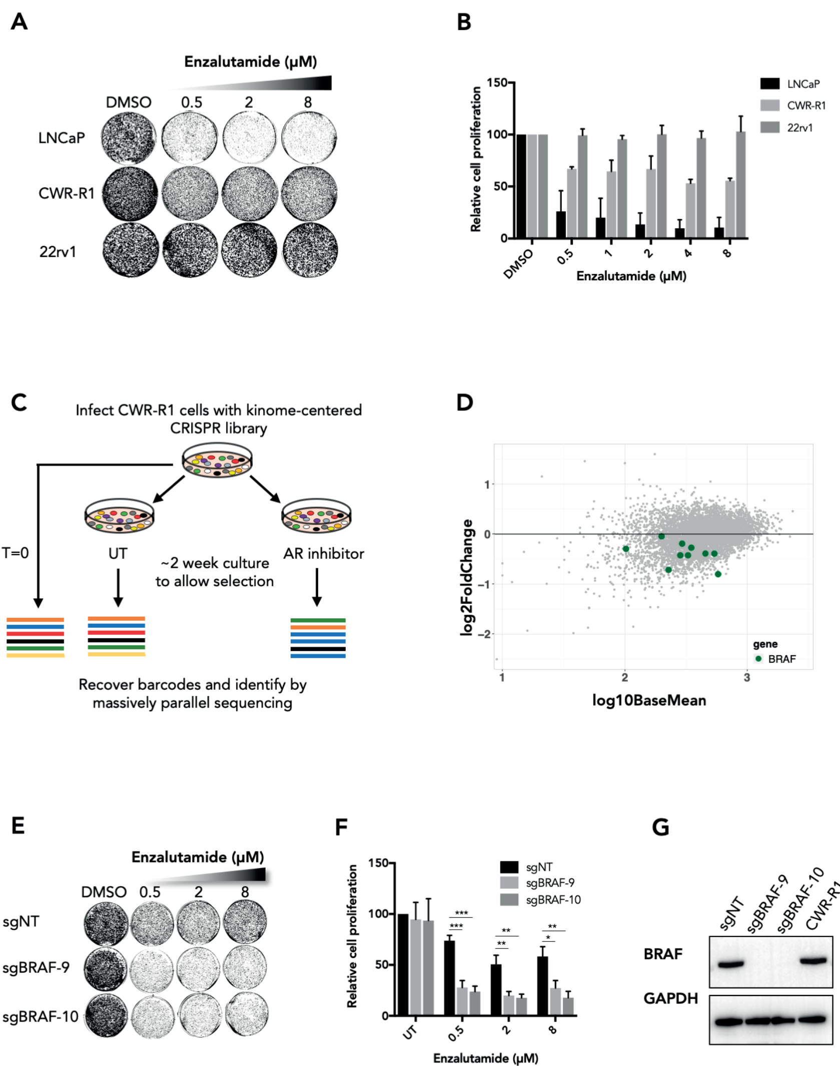

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Figure 1: A kinome-centered CRISPR-Cas9 screen identifies *BRAF* as a modulator of enzalutamide sensitivity in CWR-R1 cells. (A). Enzalutamide sensitivity of prostate cancer cell lines LNCaP, CWR-R1 and 22rv1 in a long-term growth assay. (B) Quantified data of the results shown in *A*. (C) Schematic representation of the kinome-centered CRISPR-Cas9 screen. (D) Representation of the relative abundance of the sgRNA barcode sequences of the screen. The y-axis shows the enrichment (relative abundance of enzalutamide treated/untreated) and the x-axis shows the average sequence reads of the untreated samples. (E) Long-term growth assay showing the enzalutamide response of CWR-R1 cells harboring sgRNAs targeting *BRAF*. Cells harboring a non-targeting sgRNA were used as a control. (F) Quantified growth data of the results shown in *E*. (G) Western blot showing the protein expression levels of BRAF in indicated cell lines which were used in the assays shown in *E* and *F*. Original western blots are presented in Supplemental Fig. S1D. For the bar graphs in *B* and *F*, showing the quantified data of the growth assays, the bars represent the average data from at least three independent experiments ± SEM. P-values are indicated with ***p<0.001, **p<0.01 and *p<0.05 (two-tailed *t*-test).

MAPK inhibition potentiates enzalutamide sensitivity in AR-driven prostate cancer cells harboring a *BRAF* mutation

Given the increased sensitivity to enzalutamide upon knockout of *BRAF*, we sequenced the *BRAF* kinase domain in CWR-R1 cells using a clinically validated, NGS-based, targeted sequencing assay. Through this approach, we identified a p.L597R mutation in the activating kinase domain of the *BRAF* gene. Sequencing of LNCaP cells using the same assay, revealed no *BRAF* alterations, consistent with previous reports for this cell line[2,17]. Next, we assessed whether the increased sensitivity of *BRAF*^{KO} CWR-R1 cells to enzalutamide could be confirmed by pharmacological inhibition of the MAPK pathway in combination with enzalutamide. Short-term and long-term growth assays showed that CWR-R1 cells were unresponsive to the RAF inhibitor LY3009120 (Fig. 2A-C). However, the combination of LY3009120 and enzalutamide resulted in strong inhibition of cell proliferation when compared to monotherapy treatment using these two inhibitors (Fig. 2A-C). The combination of enzalutamide and dabrafenib was also tested and found to be more effective than single drug treatments (Supplemental Fig. S2A-B), but to a lesser extent when compared to the combination of enzalutamide and LY3009120. These findings are consistent with the reduced efficacy of BRAF V600E-targeting drugs, such as dabrafenib, in non-V600E *BRAF* mutant cancer cells[18].

Pharmacological inhibition of the MAPK pathway downstream of BRAF was performed using trametinib, a MEK inhibitor, and sch772984, an ERK inhibitor. Trametinib monotherapy did not affect cell proliferation in short-term and long-term growth assays with concentrations up to 500 nM. However, when combined with enzalutamide, a strong inhibitory effect on growth was observed (Fig. 2A, 2D-E). Similar results were obtained with the ERK inhibitor sch772984, which showed a strong synergistic effect only when used in combination with enzalutamide (Fig. 2A, 2F-G). Growth assays using low con-

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centrations of enzalutamide showed that co-treatment with MAPK inhibitors enhanced enzalutamide efficacy, making the drug effective in the nanomolar (nM) range, though the effect was strongest at 0.5 μM (Supplemental Fig. S2C-H).

Although LNCaP cells do not contain an activating *BRAF* mutation, other oncogenic alterations may cause MAPK pathway activation in these cells. However, inhibition of AR in combination with MEK or ERK inhibitors in this cell line did not result in increased sensitivity to enzalutamide (Supplemental Fig. S3A-D). These result are concordant with the absence of activating MAPK alterations[2,17]. LNCaP cells are *PTEN*-deficient and were shown to be more dependent on PI3K signaling upon enzalutamide-mediated AR inhibition, through reciprocal feedback regulation of the AR and PI3K pathway. It was shown that co-targeting of the PI3K and AR pathway in LNCaP cells resulted in strong anti-tumor effects when compared to single drug treatment[8].

We also tested the combination of AR and RAF/MAPK inhibitors in two enzalutamide-resistant cell lines: AR-negative PC-3 cells and the 22rv1 cell line. In 22rv1 cells, enzalutamide resistance was shown to be mediated by the AR-V7 splice variant[19]. Both cell lines did not respond to enzalutamide, as expected. Addition of inhibitors targeting MAPK pathway components RAF, MEK and ERK did not affect the enzalutamide response of PC-3 cells (Supplemental Fig. S4A-C), excluding off-target effects of the used inhibitors. We found that 22rv1 cells showed modest sensitivity to MAPK pathway inhibitors (Supplemental Fig. S5A-C). MAPK inhibitor treatment had no effect on the enzalutamide response (Supplemental Fig. S5A-C), and knockout of *BRAF* did not confer enzalutamide sensitivity in these cells (Supplemental Fig. S5D-F). The lack of synergy between AR and MAPK inhibitors is most likely caused by the fact that enzalutamide is incapable of targeting the AR-V7 splice variant that drives resistance in 22rv1 cells, as AR-V7 lacks the ligand-binding domain.

Next, we biochemically investigated MAPK pathway activation in response to AR and MAPK inhibition as monotherapy and when used in combination in CWR-R1 cells. We found that upon enzalutamide treatment, MAPK signaling is upregulated, as shown by increased p-ERK and p-RSK levels (Fig. 2H)[20]. Inhibition of the MAPK pathway by LY3009120 or trametinib significantly downregulated p-ERK and p-RSK levels. When cells were treated with enzalutamide in addition to LY3009120, MAPK signaling was slightly increased, but still significantly lower when compared to enzalutamide monotherapy (Fig. 2H). This upregulation as a result of enzalutamide treatment was not observed when cells were treated with the combination of enzalutamide and trametinib (Fig. 2H). Together, these data suggest that MAPK inhibition in combination with enzalutamide may be effective in AR-driven prostate cancer cells having an activated MAPK pathway, through an activating *BRAF* mutation.

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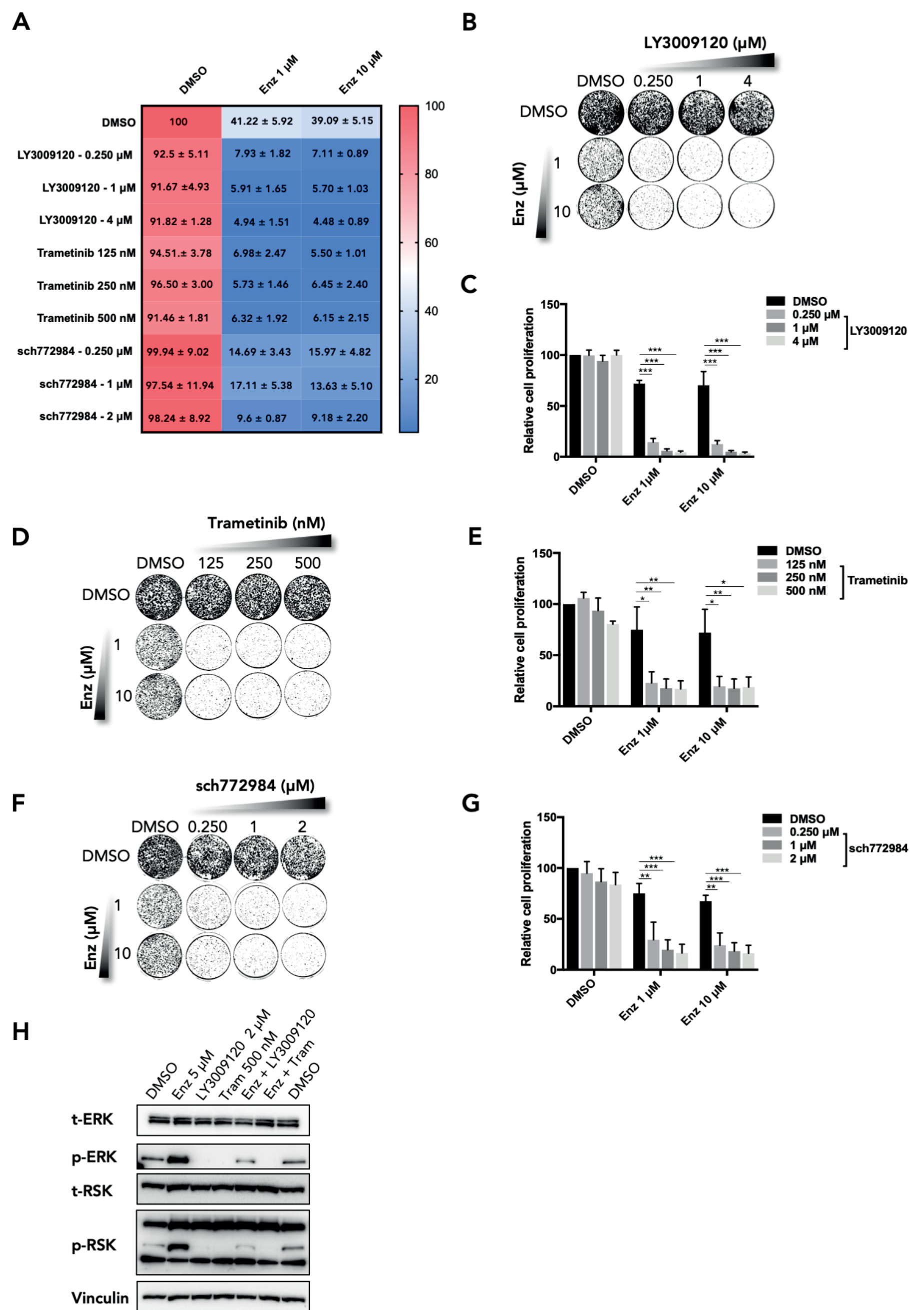

Figure 2: Pharmacological validation of screen hit *BRAF* in CWR-R1 prostate cancer cells. (A) Short-term growth assay for CWR-R1 cells treated with enzalutamide and MAPK pathway inhibitors. The percentage of growth relative to the untreated control is shown, with the standard error of the mean (SEM) for n=3 experimental replicates. (B-C) Long-term growth assay of CWR-R1 cells treated with AR inhibitor enzalutamide

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and RAF inhibitor LY3009120 as monotherapy or in combination. (D-E) Long-term growth assay of CWR-R1 cells cultured in the presence of enzalutamide and MEK inhibitor trametinib as indicated. (F-G) Long-term growth assay of CWR-R1 cells treated with enzalutamide in the presence of ERK inhibitor sch772984 at indicated concentrations. (H) Western blot showing the expression levels and phosphorylation status of MAPK pathway components in CWR-R1 cells treated with inhibitors as indicated. Vinculin was used as a loading control. Original western blots are shown in Supplemental Fig. S6A.
For the bar graphs showing the quantified data of the growth assays, the bars represent the average data from at least three independent experiments ± SEM. P-values are indicated with ***$p<0.001$, **$p<0.01$ and *$p<0.05$ (two-tailed *t*-test).

Clinical response to enzalutamide in *BRAF* mutant CRPC patients
Although infrequent, prostate cancers harbor *BRAF* mutations in around 2% of cases (Supplemental Fig. S7A)[21,22], mostly involving hotspot mutations p.K601E and p.G469A (Supplemental Fig. S7B). To explore the role of *BRAF* mutations in primary resistance to enzalutamide in CRPC patients, we analyzed sequencing data from a biopsy study (CPCT-02) at our center to identify *BRAF*-mutated patients. Biopsies were collected prior to enzalutamide or abiraterone treatment, and sequenced by either exome or whole genome sequencing[23]. We identified two patients harboring a *BRAF* p.K601E mutation. Both of these patients showed early clinical progression after commencing enzalutamide treatment (Fig. 3A-B). Out of the 30 similarly treated patients in the cohort, having clinical and PSA data available, 77% (n=23) showed a decline of ≥ 50% in PSA levels in the first three months of enzalutamide or abiraterone treatment. After 6 months of treatment, 83% (n=25) of patients had a lower PSA level compared to baseline (Supplemental Fig. S7C). These numbers are concordant with previously reported rates of primary resistance in enzalutamide-treated patients[3, 12]. Our findings suggest that activating mutations in *BRAF* are associated with primary resistance to enzalutamide, though confirmation in a larger cohort of BRAF-mutated prostate cancer patients is needed.
Together, we find that co-inhibition of the AR and MAPK pathway activity is synergistic in prostate cancer cells carrying a *BRAF* mutation. Furthermore, the poor clinical response to enzalutamide in two CRPC patients harboring *BRAF* mutations indicates that oncogenic mutations in the kinase domain of *BRAF* may result in primary resistance to therapy, which could be addressed by co-treatment with MAPK inhibitors. These findings warrant further investigation of *BRAF* alterations in the context of the enzalutamide response in larger cohorts, to further elucidate the clinical relevance of activating *BRAF* mutations in AR inhibitor-treated patients.

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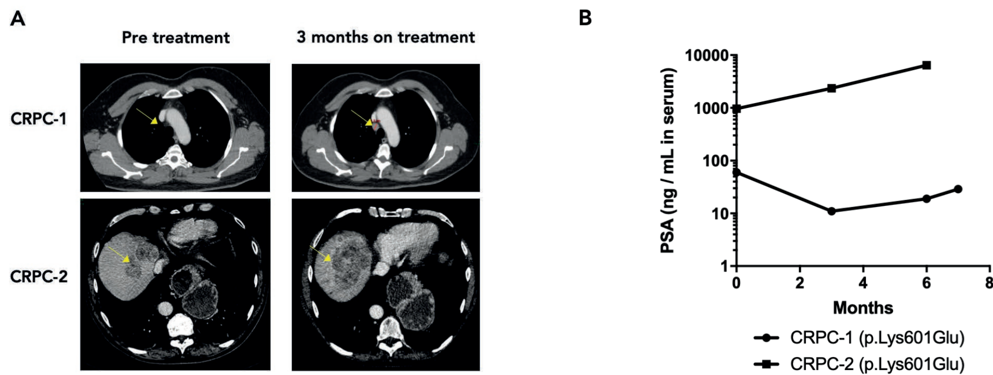

Figure 3: Enzalutamide response in two patients with *BRAF* mutant tumors. (A) Computed tomography (CT) imaging for two patients with tumors harboring the *BRAF* K601E mutation prior to starting treatment (left) and 3 months on treatment (right). The yellow arrow indicates a mediastinal lymph node in CRPC-1 and liver metastases in CRPC-2. (B) On-treatment PSA levels for the two patients shown in *A*.

Discussion

AR antagonists, such as enzalutamide, are effective in the treatment of AR-driven prostate cancer[3]. Still, resistance to AR inhibitors commonly arises during therapy, and primary resistance occurs in 10-20% of patients[3,12]. To improve treatment outcome, more insight into the primary resistance mechanisms is essential and may lead to the development of new treatment avenues.

In our study, we employed a kinome-centered CRISPR-Cas9 screen to identify genes that can be targeted to improve sensitivity to AR inhibition. We found that genetic knockout of the *BRAF* gene resulted in increased sensitivity to enzalutamide in CWR-R1 cells. These findings were confirmed through pharmacological inhibition of BRAF, or downstream components of the MAPK pathway. Through genetic profiling of CWR-R1 cells, the *BRAF* p.L597R mutation was identified, potentially conferring specific vulnerability to BRAF inhibition in CWR-R1 cells. The clinical significance of *BRAF* p.L597R has been shown in melanoma patients, where expression of this mutant was associated with sensitivity to MEK inhibitors[24, 25]. Moreover, knockdown experiments comparing WT *BRAF* to several mutant forms of *BRAF*, including p.L597R, demonstrated the oncogenic function of this *BRAF* mutant in non-small cell lung cancer (NSCLC) cells[26]. In CWR-R1 cells, increased BRAF activity as a result of this mutation may be responsible for the moderate sensitivity of these cells to enzalutamide, and their sensitivity to MAPK inhibition in combination with AR blockade. The fact that no mutations affecting *BRAF* are

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present in LNCaP[2,17], may explain the lack of synergy of combined AR/MAPK inhibition in this cell line.

Driver mutations in *BRAF* are found in a variety of cancers and are characterized by activating hotspot mutations in the kinase domain of the gene, most notably the V600E mutation[27,28]. Cancers harboring *BRAF* mutations are often sensitive to BRAF inhibitors, such as dabrafenib or vemurafenib[28]. Sensitivity can be increased by combination with MEK inhibitors, such as trametinib[29]. *BRAF* mutations in prostate cancer are rare but do occur in around 2% of patients[21,22], predominantly involving hotspot mutations in the activating kinase domain (p.K601E and p.G469A; Supplemental Fig. S7A-B)[21,22,30]. In our study, we describe two mCRPC patients with tumors harboring a p.K601E *BRAF* mutation with early disease progression after commencing enzalutamide treatment, indicating potential relevance of these mutations in driving prostate cancer cell growth and enzalutamide resistance. Validation of these findings in larger cohorts is needed to confirm whether presence of these mutations correlates with primary resistance in patients. Our findings suggest that co-inhibition of AR and BRAF in *BRAF*-mutant prostate cancer patients could be particularly effective.

Alterations in the MAPK pathway are observed in about 40% of primary and 90% of metastatic prostate cancer cases[2]. Amplification of MAPK components is frequent, while mutations in members of this pathway are less common in prostate cancer[2,31]. Whereas the clinical significance of *BRAF* mutations has been demonstrated in various disease settings, it is unclear what proportion of the MAPK alterations found in prostate cancer lead to meaningful activation of the MAPK pathway. Emerging evidence suggests that targeting the MAPK pathway may represent a viable treatment approach for advanced prostate cancer cells fully resistant to enzalutamide[31,32].

In conclusion, the findings by our group and others warrant further investigation of combined inhibition of the MAPK and AR pathway at an early stage of systemic treatment of AR-driven prostate cancer to overcome primary or acquired resistance. However, investigation of the clinical progression and longitudinal biochemical responses of enzalutamide-treated patients in larger cohorts is needed to further validate our findings in a clinical setting. The increase in availability of genetic profiling for cancer mutations in clinical settings[33] may aid further exploration of the potential for combined BRAF/AR inhibition in *BRAF*-mutant prostate cancer.

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Materials and Methods

Cell culture and generation of knockout cells

The human prostate cancer cell lines LNCaP, CWR-R1, 22rv1 and PC3 were a kind gift from Prof. W. Zwart (Netherlands Cancer Institute). All prostate cancer cell lines were maintained in RPMI. HEK293T cells were obtained from ATCC and were cultured in DMEM. Medium was supplemented with 10% FBS (Serana) and 1% penicillin/streptomycin. Cells were maintained at 37 °C in 5% CO_2. All cell lines were STR profiled. Control and *BRAF*^{KO} cells were created by infecting target cells with lentiviral particles containing LentiCRISPR v2.0 harboring non-targeting or *BRAF*-targeting gRNAs, which were cloned into the vector using Gibson Assembly (NEB cat#: E2611S) utilizing BsmBI restriction sites. For gRNA sequences see Supplemental Table S3. For virus production, HEK293T were co-transfected with lentiviral CRISPR constructs, using PEI. Target cells were seeded 1 day prior to infection. Lentiviral supernatant was added to the medium along with 5 µg/ml polybrene. Infected cells were selected with 2 µg/ml puromycin.

CRISPR-Cas9 kinome-centered dropout screen

CWR-R1 cells were infected with lentiviral particles containing the NKI Human Kinome CRISPR Knockout library at low M.O.I. (~0.2) for single viral integration, at a ~500 fold coverage, and cultured in the presence of vehicle or 10 µM enzalutamide for ~2 weeks. Barcodes were recovered and sequenced as described[11] . For sequence depth normalization a relative total size factor was calculated for each sample, by dividing the total counts of each sample by the geometric mean of all totals. After normalization, a differential test between the treated and untreated condition for each sgRNA was performed using DESeq2[15]. The output from the DESeq2 analysis contains the DESeq2 test statistic. Positive DESeq2 test statistic indicate positive log2FoldChange value, negative DESeq2 test statistic indicate negative log2FoldChange value. We sorted the output of DESeq2 on the test statistic in increasing order, putting the most significant depleted sgRNA at the top. We then used the MAGeCK[16] Robust Rank Algorithm to determine for each gene if its sgRNAs are enriched towards the top of the result list. The resulting enrichment p-values were corrected for multiple testing using the Benjamini-Hochberg correction, resulting in a FDR value. As hits we considered the genes with a FDR rounded on two decimals <= 0.1.

Proliferation assays

Colony formation assays were performed as previously described[11]. Enzalutamide, LY3009120, Dabrafenib, Trametinib, sch772984 were obtained from Medkoo Biosciences, all drugs were dissolved in DMSO and stored at -20C. Used seeding densities were 20,000 (LNCaP) or 10,000 (22rv1, CWR-R1) cells/well in 6-well plates, and drugs were

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added as indicated the next day. For 12-well assays, the used seeding densities were 10,000 (LNCaP), or 5000 (CWR-R1, 22rv1, PC3). The growth medium, containing vehicle or drugs, was refreshed every 36-48 hours. After 12–14 days of growth in presence of the drugs, when the control cells reached ~90% confluency, all cells were fixed in 2% formaldehyde and stained with 0.1% crystal violet.
For quantification of the growth assays, crystal violet was extracted by incubating the stained plates with 5% acetic acid for 1 hour at room temperature. The solution, containing the crystal violet, was transferred to a 96-well plate and measured using the Envision 2104 Multilabel Reader (PerkinElmer). Growth assays were performed at least three times for each experiment. Therefore, when quantified data is shown, bars represent the average data from at least three independent experiments ± SEM. P-values are indicated with ***p<0.001, **p<0.01 and *p<0.05 (two-tailed *t*-test).

Protein lysate preparation and western blot

Typically, CWR-R1 cells were plated at a density of 200,000 cells in per well in 6-well plates and cultured in the presence of drugs as indicated for 5 days before harvesting. Samples were prepared and western blot was performed as described previously[11], using the spectra Multicolor Broad Range Protein Ladder. Antibodies directed against BRAF (14814), GAPDH (5174), t-ERK (9102), p-ERK (4377), t-RSK (8408) were purchased from Cell Signaling; antibody against p-RSK (04-419) was purchased from Millipore; antibody targeting Vinculin (V9131) was purchased from Sigma.

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Author contributions

MvH supervised the project and acquired funding. SP and MvH conceptualized the project, methodology, interpreted the results and edited the manuscript. SP designed the experiments and wrote the initial draft. SP and JvD carried out the experiments, and JvD edited the manuscript. RB, DV and CL performed computational analyses, interpreted the data and provided bioinformatics support. RB, AMB, SL and WZ provided critical feedback on the results throughout the project and edited the manuscript. All authors reviewed the manuscript.

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Competing interests.
WZ and AMB receive research support from Astellas Pharma. The other authors declare no competing interests.

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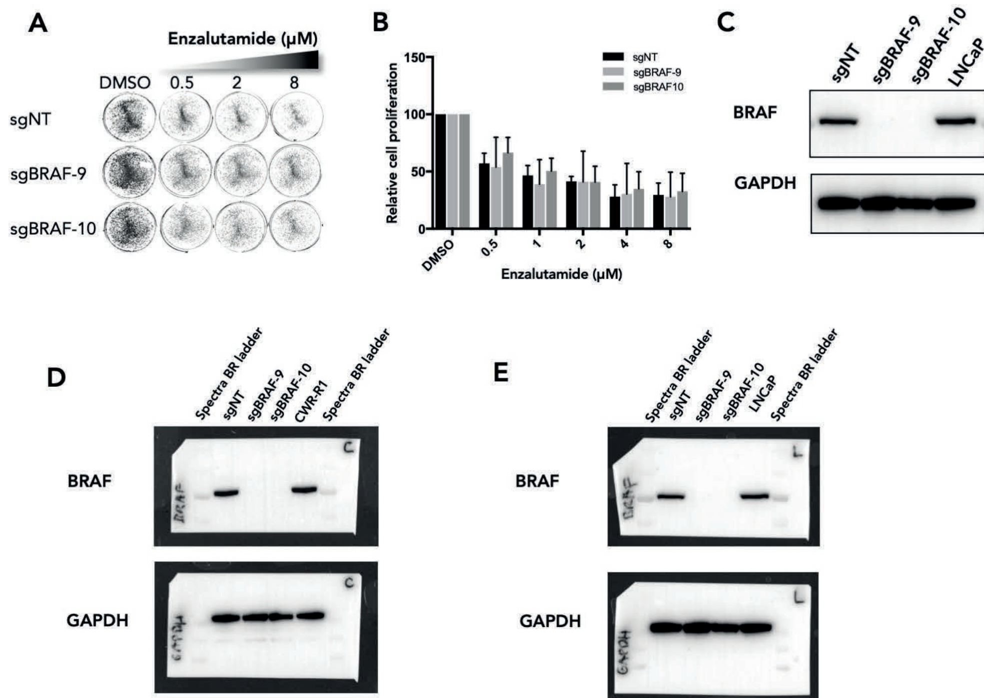
Supplementary Figures



Supplemental Figure S1: (A) Long-term growth assay for LNCaP cells harboring control or *BRAF*-targeting sgRNAs, cultured in the presence of vehicle or enzalutamide as indicated. (B) Quantified data for the results shown in *A*. Bars represent the average data from at least three independent experiments, error bars represent SEM. (C) Western blot showing protein expression levels for BRAF and GAPDH in control and LNCaP *BRAF*^{KO} cells. (D-E) Original western blot data for Fig. 1G and Supplemental Fig. 1C, respectively.

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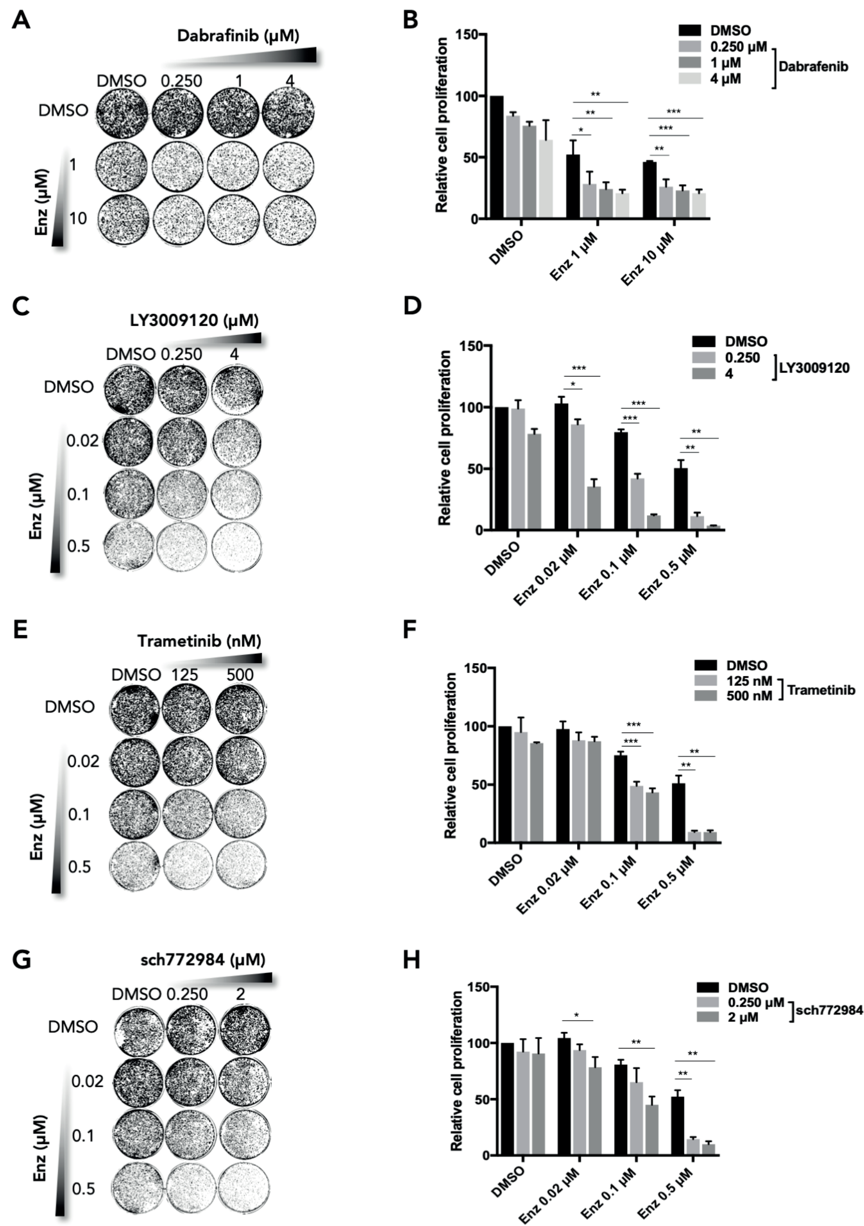

Supplemental Figure S2: (A-H) Long-term growth assays for CWR-R1 cells treated with indicated inhibitors are shown (left panels) together with quantification of these results (right panels) for n=3 experimental replicates. Bars represent the average data from at least three independent experiments, error bars represent SEM. P-values are indicated with ***p<0.001, **p<0.01 and *p<0.05 (two-tailed *t*-test).

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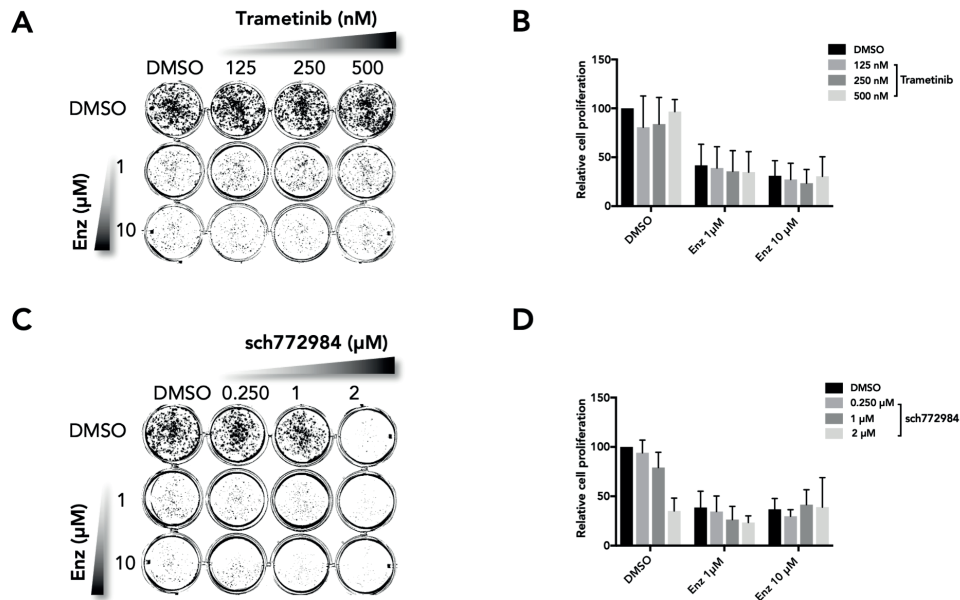
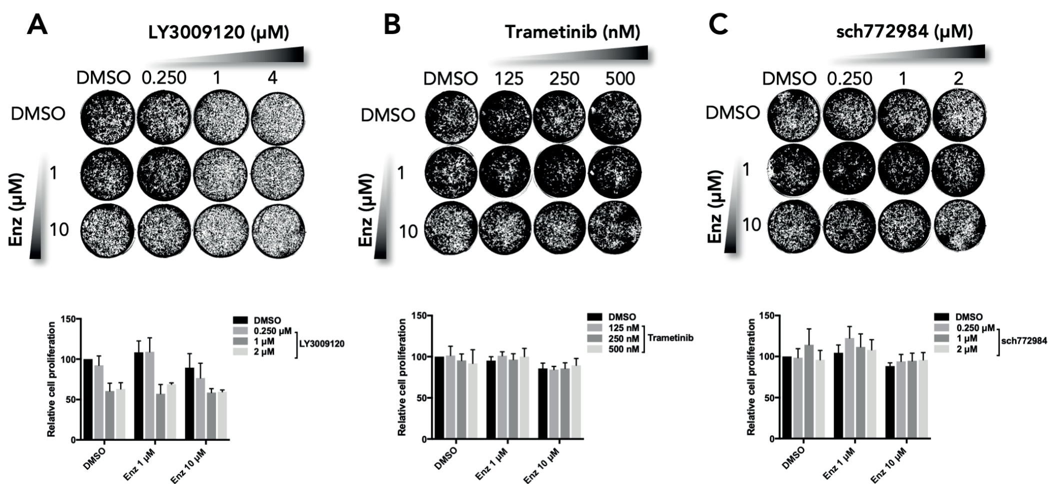


Supplemental Figure S3: (A-D) Long-term growth assays for LNCaP cells cultured in the presence of indicated inhibitors are shown (left panels), together with quantified results for n=3 experimental replicates (right panels). Bars represent the average data from at least three independent experiments, error bars represent SEM.



Supplemental Figure S4: (A-C) Long-term growth assays for PC3 cells treated with indicated inhibitors (top panels), together with the quantified results for n=3 experimental replicates (bottom panels). Bars represent the average data from at least three independent experiments, error bars represent SEM. P-values are indicated with ***p<0.001, **p<0.01 and *p<0.05 (two-tailed *t*-test).

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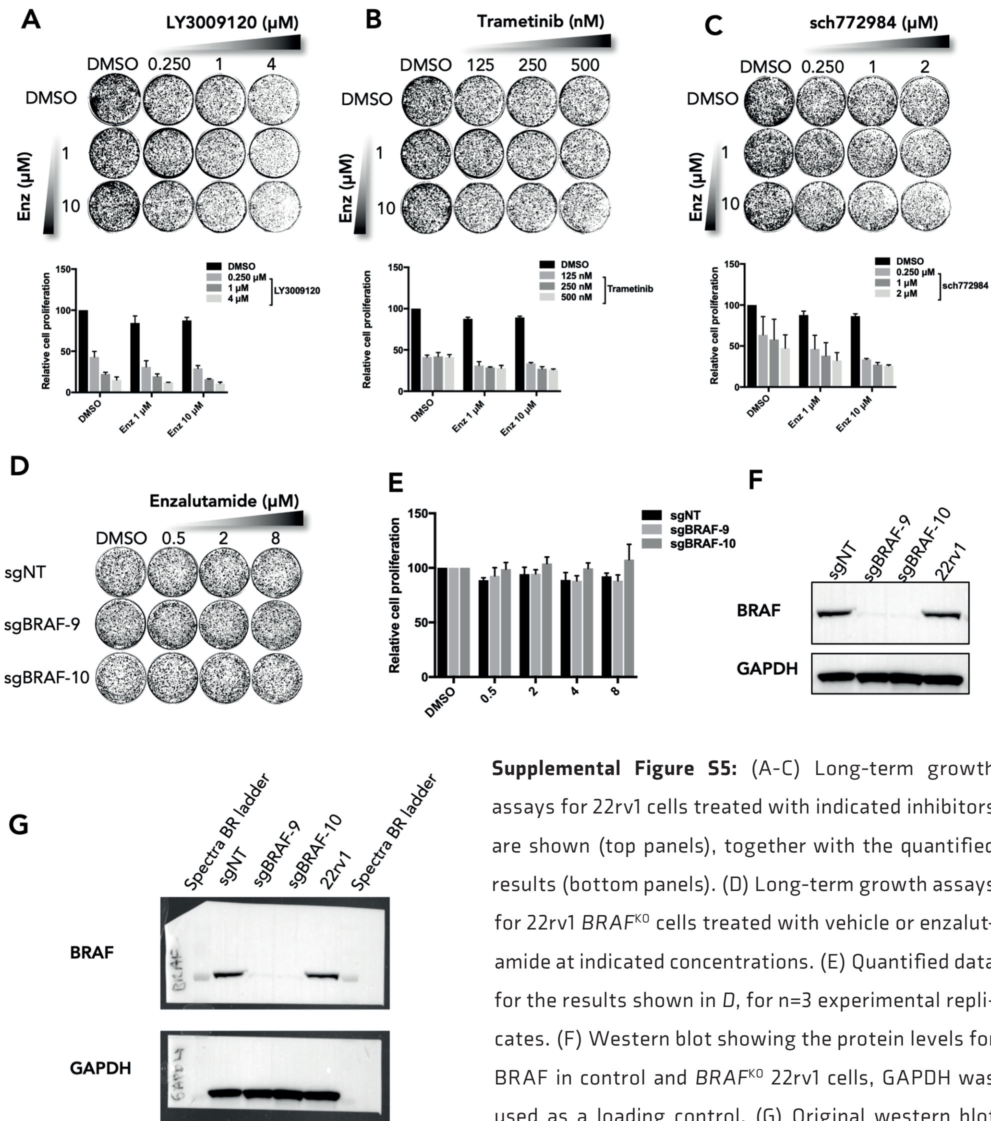

Supplemental Figure S5: (A-C) Long-term growth assays for 22rv1 cells treated with indicated inhibitors are shown (top panels), together with the quantified results (bottom panels). (D) Long-term growth assays for 22rv1 *BRAF*^{KO} cells treated with vehicle or enzalutamide at indicated concentrations. (E) Quantified data for the results shown in *D*, for n=3 experimental replicates. (F) Western blot showing the protein levels for BRAF in control and *BRAF*^{KO} 22rv1 cells, GAPDH was used as a loading control. (G) Original western blot data for Supplemental Fig. S5F. For the bar graphs in *A-C* and *E*, showing the quantified data of the growth assays, the bars represent the average data from at least three independent experiments with error bars showing the SEM.

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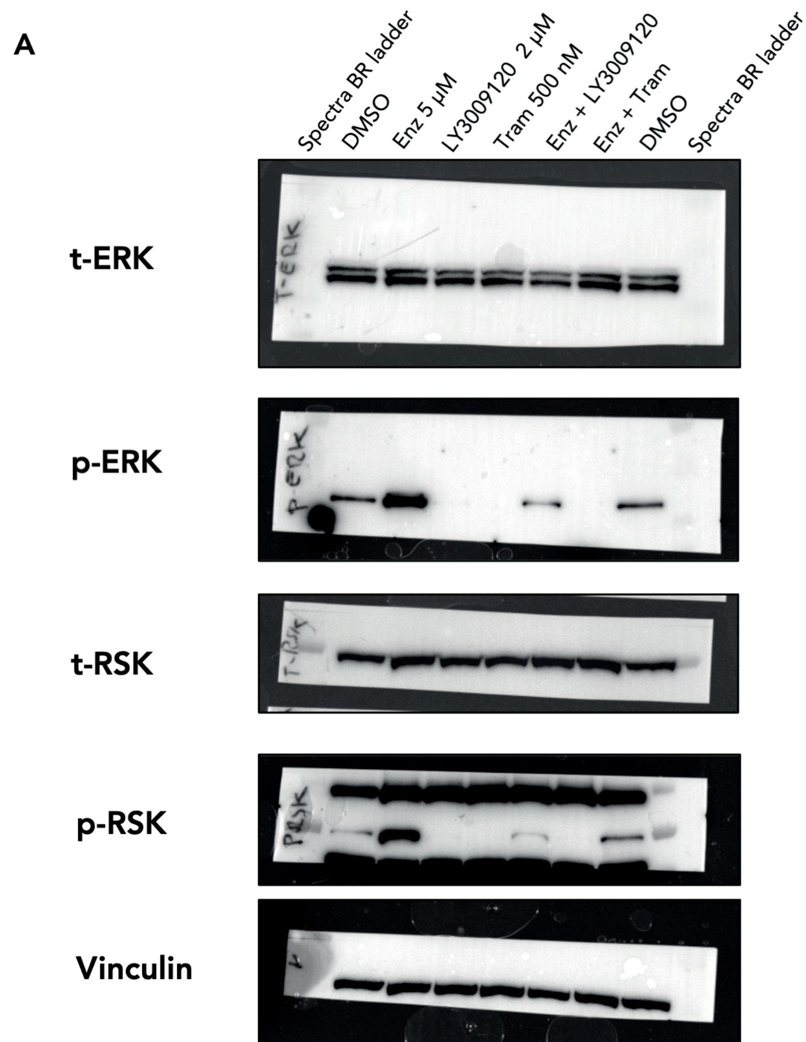

Supplemental Figure S6: (A) Original western blot data for Fig. 2H, showing the protein levels and phosphorylation status of MAPK components ERK and RSK in CWR-R1 cells cultured with drugs as indicated. Vinculin was used as a loading control.

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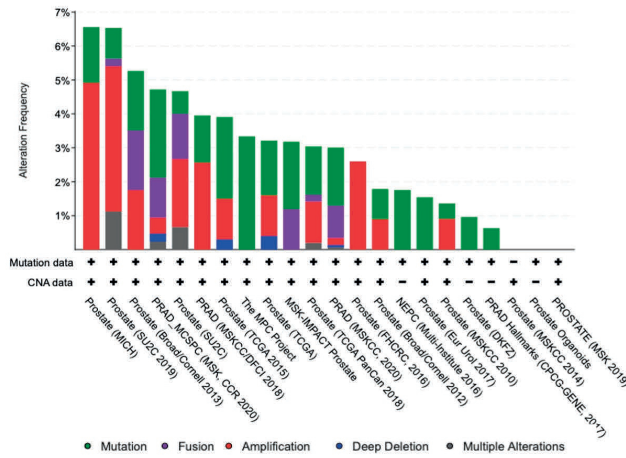

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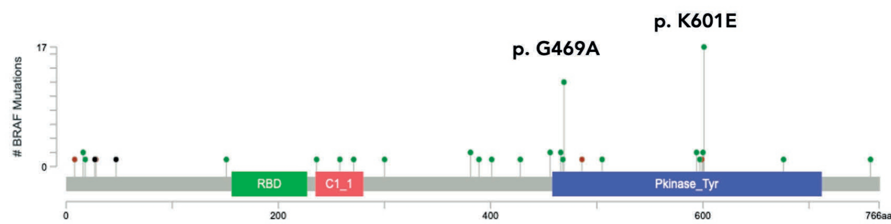

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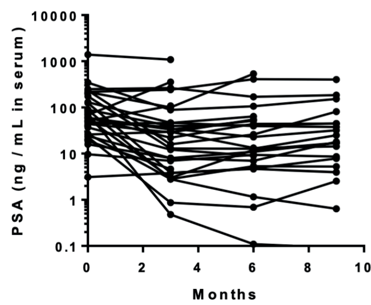
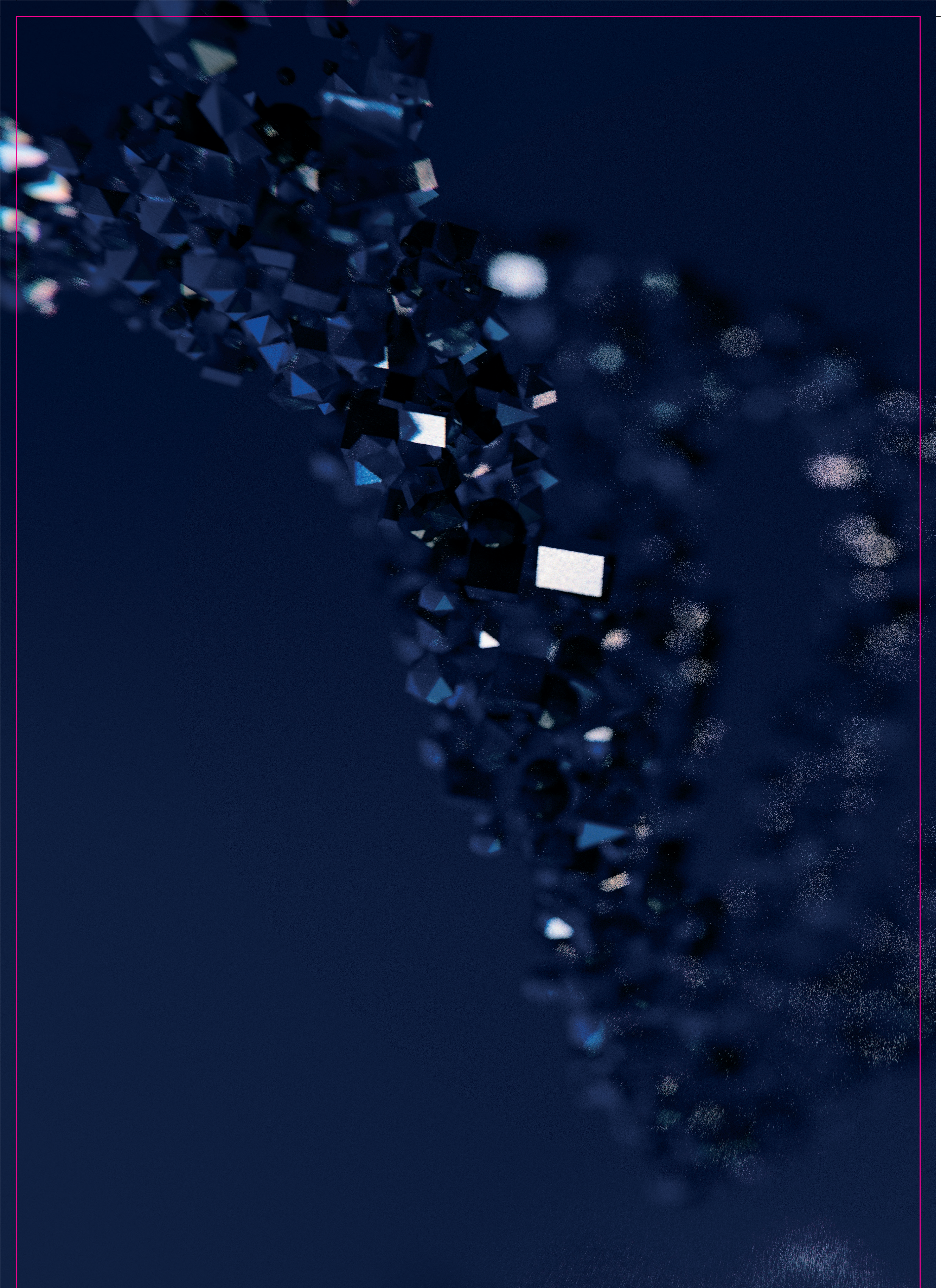


Supplemental Figure S7: (A) Overview of *BRAF* alterations in prostate cancer found in indicated studies[21,22]. (B) Overview of the frequency and nature of *BRAF* mutations in prostate cancer found in the studies indicated in *A*. (C) PSA response from enzalutamide-treated patients (n=30), harboring WT BRAF tumors, of the CPCT-02 cohort with available on-treatment PSA samples.



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Chapter 4

Molecular characterization of prostate cancer reveals MMR deficiency and alterations in *PI3K* and *RB1* associate with metastatic organotropism

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In preparation

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Abstract

The site of metastasis is associated with prostate cancer patient survival, emphasizing the need to better understand prostate cancer metastatic organotropism. Molecular profiling of 326 prostate cancer metastases revealed genetic determinants associated with organ-specific metastasis. We found that *RB1* and *PIK3CA* alterations were enriched in liver and lymph node metastases, respectively, compared to other metastatic sites. Analysis of aggregated pathway alteration data revealed a trend for overrepresentation of DNA repair and PI3K pathway alterations in lymph node compared to bone metastases. Furthermore, we observed a higher tumor mutational burden (TMB) in liver and visceral metastases than in bone and lymph node metastases. The increased TMB in liver and visceral samples was associated with an MMR-deficiency mutational signature. Half of the liver metastases with high TMB were characterized by alterations in *MSH6 (3/6)*, *MLH1 (3/6)*, and a third showed alterations in *POLD3 (2/6)*. In contrast, high TMB visceral metastases predominantly showed alterations in *MSH2* (3/4) and *POLD1* (2/4). Together, our findings implicate high TMB/MMR-deficiency as a characteristic feature of liver and visceral metastases, potentially impacting disease progression and therapy response.

Introduction

Prostate cancer is the second most common malignancy in men, with worldwide over 1.3 million new cases and around 359,000 cancer-related deaths each year (Bray et al., 2018). Localized prostate cancer has an excellent prognosis, illustrated by a 5-year cause-specific survival rate of nearly 100% (DeSantis et al., 2014). However, it is generally incurable when the disease has metastasized (Ziaee et al., 2015; Pascale et al., 2017; Deng et al., 2019). Virtually all metastatic prostate tumors regress upon androgen deprivation therapy (ADT), which targets the androgen receptor (AR) signaling pathway by lowering the amount of circulating androgens to castrate levels (Perlmutter et al., 2007; Crawford et al., 2018; Karantanos et al., 2013). Unfortunately, metastatic prostate cancer will invariably progress to metastatic castration-resistant prostate cancer (mCRPC), which is associated with high morbidity and mortality. mCRPC is driven by deregulated AR signaling, mediated through *AR* amplification, *AR* mutations, or the emergence of constitutively active AR splice variants (Beer et al., 2013; Karantanos et al., 2013).
Approximately 90% of patients with mCRPC will develop bone metastases during the course of the disease (Bubendorf et al., 2000; Gandaglia et al., 2014; Budczies et al., 2015; Shou et al., 2018), making bone the most common site for prostate cancer metastases. Liver, lymph node (LN), and visceral metastases are less frequent and commonly

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occur in combination with bone metastases (Gandaglia et al., 2014; Halabi et al., 2014; Halabi et al., 2016; Shou et al., 2018). Importantly, there is a strong association between the site of metastasis and the prognosis of patients with disseminated prostate cancer. Patients with only LN metastases have the most favorable outcome, while patients with liver metastases have the worst prognosis, irrespective of concurrent metastases at other sites (Pond et al., 2014; Gandaglia et al., 2014; Halabi et al., 2014; Gandaglia et al., 2015; Halabi et al., 2016; Shou et al., 2018).

Cancer metastasis to specific organs occurs through a non-stochastic process termed "metastatic organotropism", which is cancer-type specific and governed by interactions between the tumor cells and the tumor microenvironment (TME) at the pre-metastatic niche (Liu et al., 2016; Gao et al., 2019). Mediators of these interactions include genetic changes that alter growth and survival signals (Jacob et al., 2015), metabolism (Pani et al., 2010; Weber et al., 2016), the expression of cell surface markers (Barthel et al., 2013), and secreted factors (Peinado et al., 2011). Mechanistic understanding of CRPC metastasis and unraveling the genetic determinants underlying metastatic organotropism may contribute to patient stratification and reveal molecular processes amenable to therapeutic exploitation, thereby improving prostate patient care.

Here, we set out to explore the molecular characteristics associated with the location of prostate cancer metastases using Whole Genome Sequencing (WGS) data from 326 mCRPC biopsies. The large number of samples included in this study enabled us to compare genetic traits of metastases from different sites with sufficient power. We investigated differential mutations, amplifications, deletions, and tumor mutational burden (TMB).

Results

Patient cohort characterization

We obtained the metastatic castration-resistant prostate cancer (mCRPC) cohort from the Center for Personalized Cancer Treatment (CPCT), a Dutch nationwide biopsy program. For each of the 326 patients, a fresh-frozen biopsy was collected from a metastatic site. Inclusion occurred at any stage of the disease, irrespective of systemic treatments received. However, all patients received androgen deprivation therapy. Of the 326 metastatic samples, 149 originated from lymph nodes, 105 from bone, 49 from liver, and 23 from visceral sites (Fig. 1A and 1B). Visceral samples comprise biopsies taken from any organ located in the peritoneal or thoracic cavity, apart from the liver, bone, or lymph nodes, thus constituting a more heterogeneous group than the other sites. Whole-genome sequencing data was acquired through standardized sequencing and bioinformatics analysis (Priestly et al., 2019; Roepman et al., 2021). We first generated an overview of the most common genetic alterations observed in the cohort (Fig. 1C).

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Our findings were consistent with a previously reported analysis of a subset of 197 samples from the same cohort (van Dessel et al., 2019), showing genetic aberrations such as *AR* amplifications, deletions and mutations in *RB1*, *TP53* and *PTEN*, and *TMPRSS2-ERG* fusions (Fig. 1C).

Moreover, among the genes in the top-5 genetic aberrations, we found amplification of *CSMD3* and *HEY1*. While *CSMD3* and *HEY1* are both on chromosome 8q, they are 33 Mb apart, making them unlikely focal copy number events. However, broader amplification of 8q has been reported before in prostate cancer (El Gammal et al., 2010).

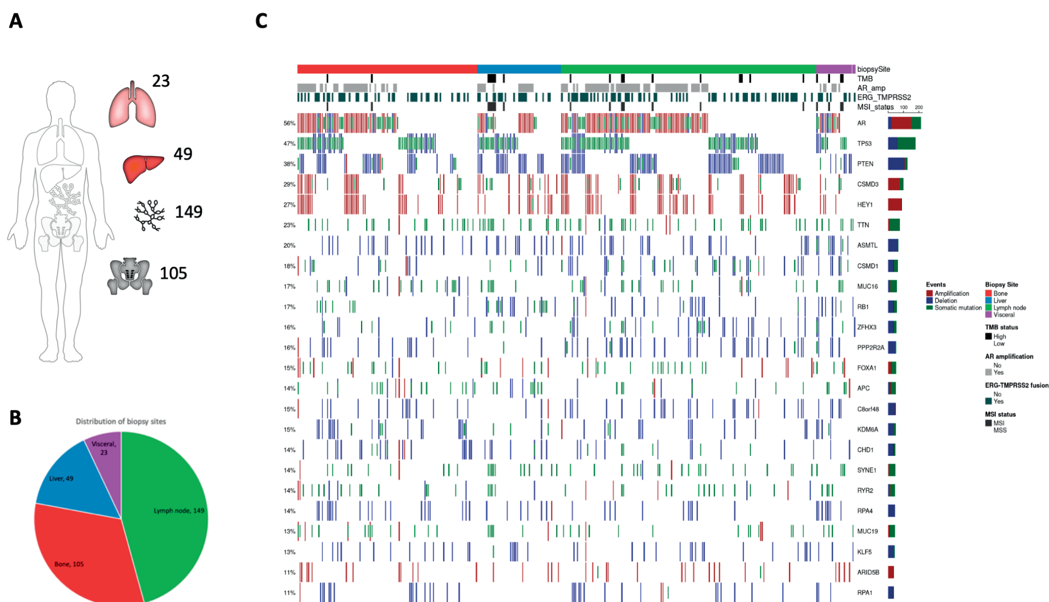

Figure 1: Genetic characterization of prostate cancer tissue from different metastatic sites (A-B) Overview of the number of biopsy samples collected from each metastatic site. (C) Oncoprint showing the most frequent mutations and recurrently deleted or amplified genes in this cohort.

Characterization of alteration frequencies in prostate cancer-relevant pathways

To increase the likelihood of identifying genetic determinants of clinical relevance, we focused on targetable prostate cancer-relevant pathways, which included AR (Augello et al., 2014; Taylor 2010), PI3K (Carver et al., 2011; Taylor et al., 2010), Wnt (Yeh et al., 2019), DNA repair (Schiewer et al., 2018) and RAS/RAF pathways (Taylor et al., 2010). We analyzed the relative frequencies of amplifications, deletions, and mutations in genes associated with these pathways comparing different sites, using a Fisher exact test and the Benjamini-Hochberg procedure (Benjamini et al., 1995) to control for mul-

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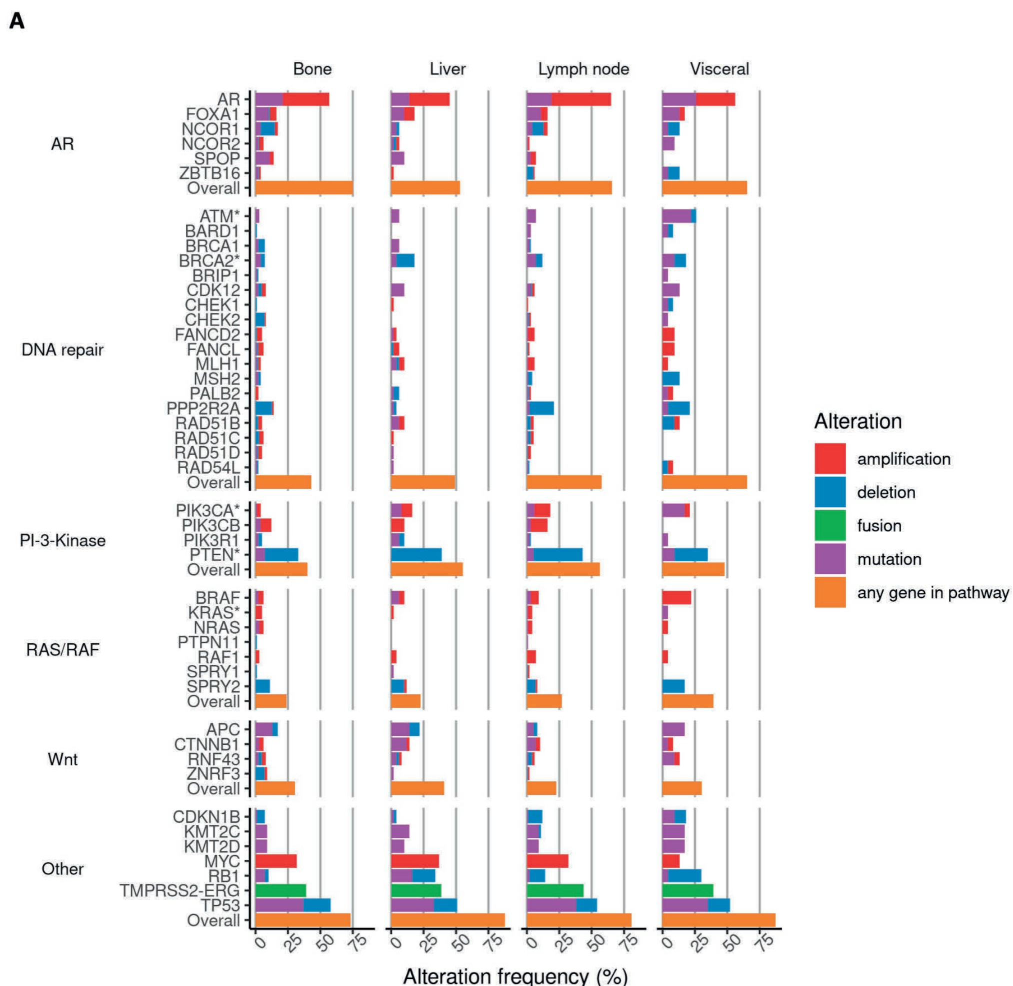
tiple testing (Fig. 2A). We found equal alteration rates for most gene alterations across the metastatic sites, but some reflect a site preference (Supplemental Table 1). The data revealed significant enrichment of *RB1* alterations in liver (35%) and visceral (30%) metastases, while much lower rates were found in bone (10%) and lymph nodes (13%) samples (p-value: 0.012). Furthermore, we found relatively fewer *PIK3CA* alterations in bone metastases (4%) than in the other sites (14-22%) (p-value: 0.034). *ATM* alterations were found in 26% of the visceral metastases, compared to 4-9% for the other sites (p-value: 0.077). An analysis of the aggregated pathway data, comparing pathways with mutations in any of its genes to its wild-type counterpart between bone and lymph node metastases, revealed a trend towards alteration enrichment affecting the DNA repair pathway (adjusted p-value: 0.066) and the PI3K pathway (adjusted p-value: 0.066) in lymph node compared with bone metastases (Fig. 2A and Supplemental Table 2).



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▲**Figure 2:** Distribution of prostate cancer-relevant alterations in common metastatic sites. (A) Overview showing the alteration frequency of genes frequently altered in common metastatic tissues in prostate cancer. In addition, the aggregated pathway data, based on alteration frequencies within these pathways is shown for the different sites.

Tumor mutation burden and mismatch repair deficiency enrichment characterize liver and visceral metastases

Next, we explored the frequency of mutations more broadly, beyond the scope of individual pathways, assessing potential differences in TMB between metastatic sites. Strikingly, we found that metastases with the highest TMB were predominantly from the liver and visceral sites (Fig. 3A). A comparison of the TMB across biopsy sites showed more mutations in liver (9.2 mut/Mbp) and visceral metastases (22.1 mut/Mbp) as compared to bone (4.1 mut/Mbp) and lymph node samples (6.0 mut/Mbp), while there was no significant difference between liver and visceral sites (Fig. 3B). Next, we explored the relationship between *RB1, ATM* and *PIK3CA* alterations and TMB (Supplemental Fig. S1). There was no significant difference in TMB in samples with or without *RB1* alterations from bone, lymph node, or visceral sites (Supplemental Fig. S1A). However, in biopsies obtained from the liver, we found that *RB1* alterations were associated with a significantly lower TMB (Supplemental Fig. S1A). In liver and visceral metastases, *ATM* alterations were associated with higher TMB when compared to bone and lymph nodes samples (Supplemental Fig. S1B). For *PIK3CA*, alterations were associated with higher TMB in lymph node samples compared to the other three sites (Supplemental Fig. S1C).
Somatic mutations may result from various mutational processes, such as errors in DNA repair pathways, DNA replication, or exogenous factors such as exposure to radiation or mutagens. These different mutational processes give rise to different genetic profiles characterized by distinct mutations and mutation types. We analyzed the samples from the different sites for characteristic mutational signatures to identify the underlying mutagenesis processes responsible for the observed phenotype, using the Catalogue of Somatic Mutations In Cancer (COSMIC) mutational signatures. These include mutational signatures for error-prone non-homologous end joining (NHEJ) and mismatch repair (MMR). Analysis of all metastatic samples revealed a strong association between TMB and the ID2 mutational signature, which is associated with small insertions and deletions linked to DNA mismatch repair deficiency (Fig. 3C). Moreover, we found TMB-high samples associated with the ID2 mutational signature to be enriched for liver and visceral metastases, explaining all but one of the hypermutant samples (Supplemental Fig. S2A). Combined, these findings are in line with the microsatellite instability (MSI) status of these samples (Fig. 1C), as MSI is a phenotype associated with a defective MMR pathway (Richman, 2015). Together, our data show that MMR deficiency is more frequent in liver and visceral metastases with high TMB, when compared to bone and lymph node metastases.

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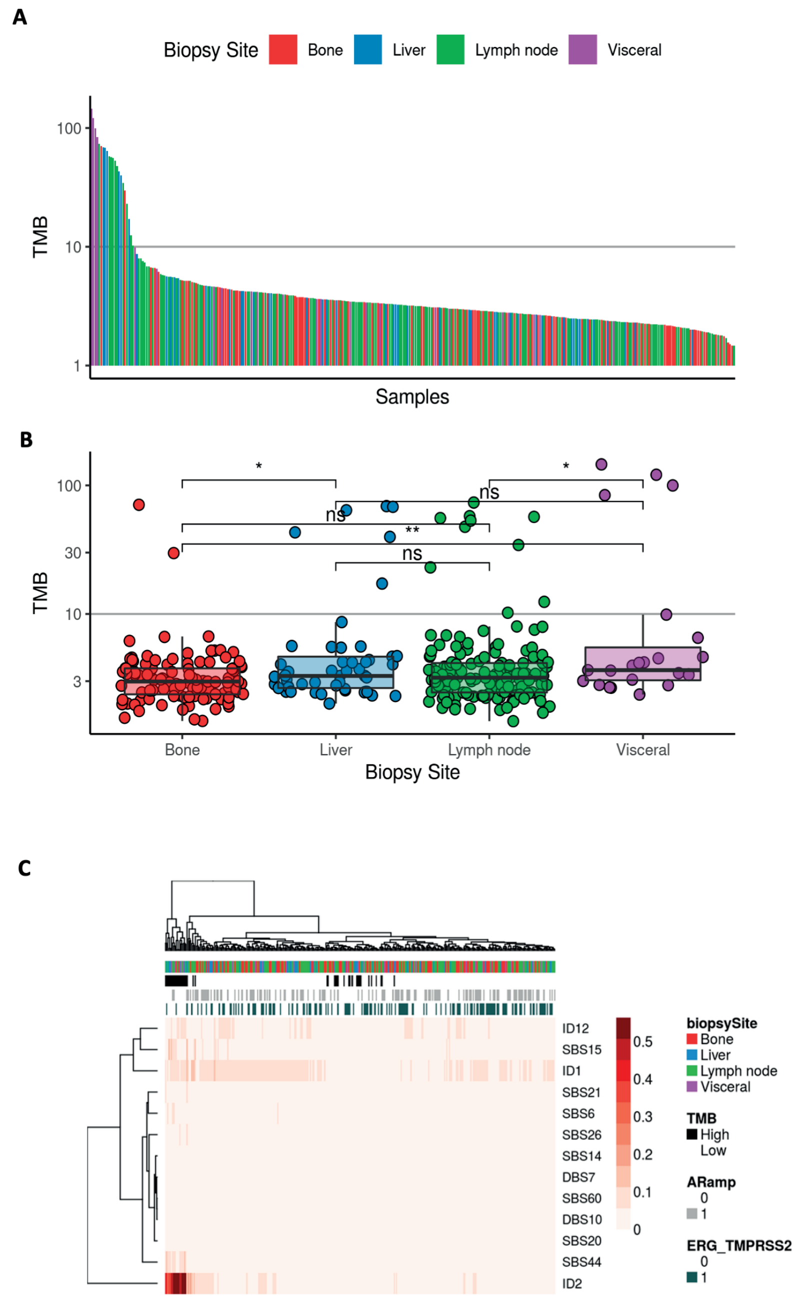

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▲**Figure 3:** Increased TMB and MMR deficiency in liver and visceral metastases. (A) Waterfall plot of all samples based on TMB. Y-axis: mutations per megabase (mut/Mbp), x-axis: hierarchical order (B) Boxplot showing the average TMB (mut/Mbp) per biopsy site. Asterisks indicate significant differences between biopsy sites. P-value indicated by *p = 0.05 (t-test). (C) Heatmap showing enrichment of samples based on the mutational signatures according to COSMIC.

Alterations in distinct MMR components characterize liver and visceral metastases with high TMB

The higher TMB and enrichment for MMR deficiency in liver and visceral metastases prompted us to explore the MMR pathway further in these samples. We analyzed the MMR machinery's key components, as defined by the KEGG mismatch repair pathway. Moreover, we added mutations in *BRCA1/2* (Messina et al., 2020: Yadav et al., 2020) and *POLE* (Yadav et al., 2020), which are associated with TMB, and genes implicated in Lynch syndrome (*MLH1*, *MSH1* and *MSH6*) that are associated with elevated prostate cancer risk (Martinez-Lopez et al., 2013; Raymond et al., 2013; Ryan et al., 2014; Haraldsdottir et al., 2014). The alteration frequency of MMR genes *MLH1* and *POLD1* was significantly overrepresented in liver and visceral metastases, respectively, compared to other sites, and found to be associated with high TMB (Fig. 4A). High TMB was also associated with *POLD3* and *MSH6* alterations in liver samples, and *MSH2* alterations in visceral metastases (Fig. 4A). Importantly, MSH2, MSH6 and MLH1 are part of the same complex involved in MMR. The MSH2 and MSH6 proteins heterodimerize to form the MutSα complex, which recognizes mismatched bases, leading to recruitment of the heterodimer complex of MLH1 and PMS2 (mutLα), initiating repair of the damage (Richman, 2015). Together, we found that alterations in MMR genes were biased towards liver and visceral metastases, with distinct genes of the same MMR pathway complex being differentially altered in liver and visceral metastases with high TMB.

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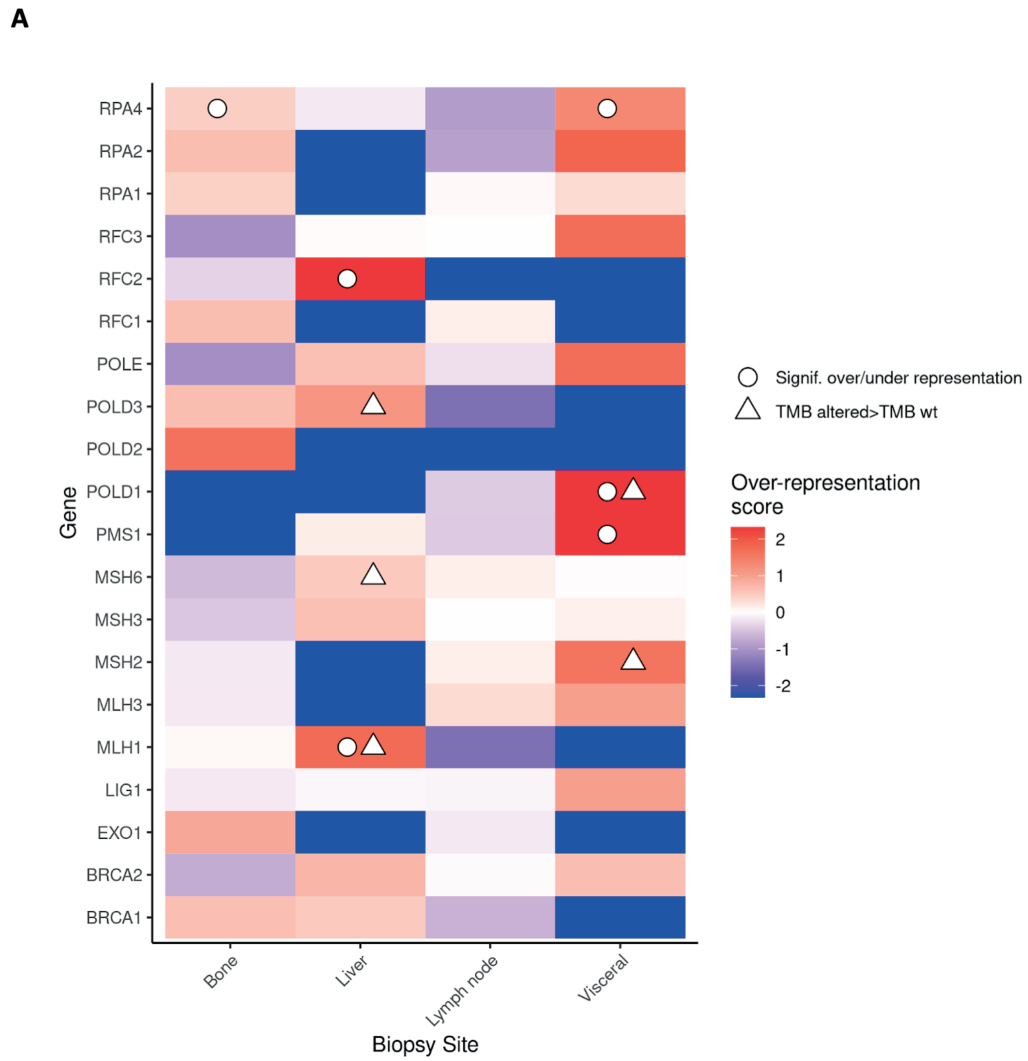

Figure 4: Key components of the MMR machinery are altered in high TMB samples. (A) Heatmap showing the alterations in key MMR genes, and *POLE* and *BRCA1/2* in high vs. low TMB samples from different metastatic sites.

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Discussion

Using the largest WGS dataset currently reported for metastatic prostate cancer, we investigated genetic differences between metastatic sites to reveal site-specific molecular characteristics. Identifying genetic traits of metastases associated with organ-specific bias may improve patient stratification, directing treatment based on the metastatic site and genetic makeup as described here.
Comparison of the alteration frequencies of genes and pathways across metastases revealed differential enrichment of *RB1* and *PIK3CA* alterations in liver and bone, respectively, compared to other sites, and a trend for overrepresentation of DNA repair and PI3K pathway alterations in lymph node compared to bone metastases. Intriguingly, we found that the TMB is higher in liver and visceral metastases compared to tumor tissue collected from LN and bone. Moreover, in liver and visceral samples, distinct MMR components were differentially altered between these two sites in high TMB samples.
It is important to note that this study does not establish a novel causal cascade for the genetic changes found and metastatic organotropism. Genetic alterations in tumor cells may i) induce systemic organotropic effects, originating from the cancer cell, that promote seeding at specific tissues, ii) confer tumor-intrinsic traits that influence tissue distribution, making them more compatible with specific tissues, or iii) be acquired at the metastatic site post-engraftment. Tumor cells have been shown to secrete factors that alter the stromal compartment at the pre-metastatic niche, dictating metastatic organotropism (Kaplan et al., 2005; Hiratsuka et al., 2006; Webber et al., 2010; Deng et al., 2012; Hoshino et al., 2015). Tumor cell-intrinsic properties arising from genetic alterations may promote metastasis and confer improved compatibility with specific tissues, regulating organ-specific metastasis. These include genetic alterations affecting various cellular processes, such as the utilization of specific growth and survival signals that influence cell fitness (Jacob et al., 2015; Sethi et al., 2011; Fournier et al., 2015), altered metabolism (Pani et al., 2010; Rodriguez-Torres et al., 2015) and immune cell interactions (Takeda et al., 2001; Eyles et al., 2010). Potentially, genetic alterations may be acquired at the site of metastasis. Future studies comparing sequencing data from primary and metastatic biopsies may reveal specific genetic changes as either novel tumor-intrinsic drivers or passenger mutations in the process of metastatic organotropism in prostate cancer.
Analysis of high TMB samples revealed an MSI phenotype and association with alterations in MMR genes *MLH1*, *MSH2* and *MSH6* in liver and visceral metastases. Alterations in the canonical MMR genes *MSH2*, *MSH6*, and *MLH1*, have been found in prostate cancer patients with primary (Guedes et al., 2017; Schweizer et al., 2016) and advanced disease (Schweizer et al., 2016; Rodrigues et al., 2018; Ryan et al., 2019; Pritchard et al., 2014; Antonarakis et al., 2019; Guedes et al., 2017). Genetic aberrations affecting MMR genes

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are rare in primary and metastatic prostate cancer. Alterations rates for *MSH2*, *MSH6*, *MLH1*, and *PMS2* are estimated to be around 1-5% in prostate cancer (Robinson et al., 2015; Guedes et al., 2017; Rodrigues et al., 2018; Sedhom et al., 2019; Antonarakis et al., 2019). A study analyzing advanced prostate tumors (n=60) found 12% (n=7) of tumors to be hypermutated, characterized by MMR alterations predominantly involving *MSH2* and *MSH6* mutations. These included biopsies from prostate, as well as liver, bone, LN, adrenal, and kidney metastases (Pritchard et al., 2014). Furthermore, evidence suggests a link between MMR deficiency and prostate cancer risk (Win et al., 2012; Rosty et al., 2014; Ryan et al., 2014; Pritchard et al., 2014; Raymond et al., 2013). The mechanisms underlying the site-specific bias observed for alterations in different MMR genes in liver and visceral metastases remain elusive. Potentially, changes in TMB and MMR-proficiency may result in genetic changes conferring tumor cell properties that promote metastasis to, or make them more compatible with, these anatomical sites.
Alterations in *POLD1* and *POLD3* in metastatic prostate cancer are rare and not well studied (Cerami et al., 2012). POLD1 and POLD3 have an essential role in maintaining genome stability and S-phase progression (Prindle et al., 2012; Tumini et al., 2016). Therefore, genetic aberrations affecting *POLD1* and *POLD3* may contribute to prostate tumorigenesis.
Our data showing enrichment for *RB1* alterations in liver metastases compared to the other tissues may have therapeutic implications. RB loss was shown to modulate the anti-hormonal therapy response in prostate cancer, diminishing the efficacy of agents targeting the AR signaling axis (Sharma et al., 2007; Nyquist et al., 2020). It was shown that combining PARP and ATR inhibition may significantly inhibit the growth of prostate tumor cells with a concurrent loss of *RB1* and *TP53*, which respond poorly to AR-directed therapy (Nyquist et al., 2020). Therefore, *RB1*-deficient metastases of the liver may respond worse to AR inhibition and better to PARP/ATR inhibition compared to bone, lymph node or visceral metastases. Furthermore, aggregated mutation data showing a trend for overrepresentation of the alteration frequency of the DNA repair and PI3K in lymph node versus bone metastases may suggest a differential response to therapeutics targeting these pathways in these tissues when compared to other sites.
Our findings showing enrichment of high TMB and MMR deficiency in liver and visceral sites may have clinically relevant implications. High TMB and MMR deficiency was shown to be predictive for immunotherapy response, owing to neoantigen expression (Goodman et al., 2017). Studies investigating immune checkpoint inhibitors ipilimumab and pembrolizumab as monotherapy in prostate cancer have shown limited efficacy in unselected patients with mCRPC (Beer et al., 2017; Kwon et al., 2014; Antonarakis et al., 2019). However, emerging evidence suggests that immunotherapy may be more effective in patients with MMR-deficient prostate cancer (Graham et al., 2020). Results from a phase II trial indicate that CRPC patients pre-treated with chemotherapy respond worse

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to the combination of nivolumab and ipilimumab compared to patients who did not receive previous chemotherapy treatment (Sharma et al., 2020). Moreover, prostate cancer metastases of the liver are associated with a poor outcome (Halabi et al., 2016; Pond et al., 2014; Shou et al., 2018). Based on our findings, future studies assessing immunotherapy in advanced prostate cancer may emphasize evaluating therapy response in a subset of patients with MMR-deficient liver and visceral metastases specifically. Potentially, patient stratification based on metastatic site associated with altered MMR status, and prior chemotherapy treatment, could improve immunotherapy response when compared to unselected patients.

Furthermore, MMR deficiency is associated with resistance to platinum-based chemotherapies such as cisplatin (Fink et al., 1997; Aebi et al., 1997; Pors et al., 2005). Even though taxanes are the cornerstone chemotherapeutic for prostate cancer (Tannock et al., 2004), studies investigating platinum-based agents suggest clinical relevance for this class of therapeutics in this context (Ross et al., 2008; Sternberg et al., 2009; Hager et al., 2016; Corn et al., 2019; Leal et al., 2019; Schmid et al., 2020). MMR-deficient liver and visceral metastases may respond worse to platinum-based agents. Therefore, future studies investigating the response to platinum-based therapies in prostate cancer patients with advanced disease may focus on assessing these metastatic sites and MMR status as factors influencing therapy response.

In summary, we found that *RB1* and *PIK3CA* were differentially altered in liver and bone metastases, respectively, compared to the other tested sites, and we observed a trend for differential alteration frequency of the DNA repair and PI3K pathway in lymph node vs. bone. We showed that high TMB and increased MMR deficiency characterized liver and visceral metastases, with distinct MMR components being altered at these two sites. Future research comparing primary and metastatic biopsies may reveal whether the genetic determinants as described here are acquired at the metastatic site, or stem from the primary tumor. High TMB/MMR deficiency may confer vulnerability to immunotherapy, potentially benefiting this subset of patients. Conversely, platinum-based therapies may perform worse in MMR-deficient metastases in the liver and visceral sites. Future studies focusing on therapeutic exploitation of DNA repair alterations and anti-tumor immunity in prostate cancer may consider metastatic site and erroneous MMR as critical factors for therapy response. This may lead to improved patient stratification based on the metastatic site and the genetic traits as described here.

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Materials and methods

Samples and genomics features

Whole-genome sequencing (WGS) data from 386 metastatic castration-resistant prostate cancer samples were obtained from the Hartwig Medical Foundation. From these, only biopsies taken from metastatic sites were included. When a patient donated two biopsies from the same metastatic site, only the earliest one was included in the analysis to avoid duplicates. In total 149 lymph node samples, 105 bone samples, 49 liver samples and 23 visceral samples were considered. Visceral was defined as a sample taken from any organ located in the peritoneal cavity except the liver.

Various genomic features were extracted from the sequencing data: somatic and germline variants, structural variants, copy number variants and mutational signatures. The tumor mutational burden (TMB) was calculated for each sample by summing over all the somatic mutations detected, using the alteration counts established by MutationalPatterns. The resulting number is then divided by the genome length, to arrive at a TMB estimate per Mb.

Somatic variants

Somatic variants were filtered on annotation impact: only variants with a moderate to high impact were included.

Structural variants

We first kept only structural variants that passed the GRIDSS quality filters : QUAL>=1000 with AS>0 and RAS>0 or QUAL>=500 with AS>0 or RAS>0 (Cameron et al., 2017)). We then restricted ourselves to determining whether samples presented an ERG-TMPRSS2 fusion.

Copy number variants

We first extracted a copy number per gene by averaging each gene's minimal and maximal copy number when genes span multiple altered segments. We then encoded amplification as genes presenting six or more copies and deletions as genes showing one copy or less.

Mutational signatures

Mutational signatures were obtained using the R package MutationalPatterns (v2.0.0) (Blokzijl et al., 2018) and all COSMIC signatures. Signature scores were calculated as the percentage of contribution of a given signature compared to all signatures.

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Gene sets

Recurrent alterations

We first looked at recurrent aberrations. Only genes altered in at least 10% of the cohort were kept. We evaluated recurrent aberrations in copy number by using RUBIC (v1.0.3) (van Dyk et al., 2016). For each recurrently aberrant region, a representative gene was extracted. This representative gene was either a known oncogene, or the first gene of the region.

Prostate cancer-relevant genes

We separately assessed genes in commonly altered prostate cancer pathways, such as the AR or PIK3CA pathway (Cancer Genome Atlas Research Network).
Full list:
AR, ZBTB16, NCOR1, NCOR2, FOXA1, SPOP, ATM, BARD1, BRCA1, BRCA2, BRIP1, CDK12, CHEK1, CHEK2, FANCD2, FANCL, MLH1, MSH2, PALB2, PPP2R2A, RAD51B, RAD51C, RAD51D, RAD54L, PTEN, PIK3CA, PIK3CB, PIK3R1, CDKN1B, KMT2C, KMT2D, MYC, RB1, TP53, APC, CTNNB1, RNF43, ZNRF3, BRAF, KRAS, NRAS, PTPN11, RAF1, SPRY1, SPRY2.

MMR-associated genes

We also studied genes associated explicitly with DNA mismatch repair. These are defined as genes pertaining to the KEGG mismatch repair pathway, to which BRCA1/2, POLE and genes involved in the Lynch syndrome were added.
Full list: MSH2, MLH1, PMS1, PMS2, MSH6, MLH3, POLE, BRCA1, BRCA2, POLD1, POLD2, RFC1, RFC3, RFC2, MSH3, POLD4, RFC4, LIG1, RFC5, RPA1, RPA3, POLD3, RPA2, PCNA, SSBP1, RPA4, EXO1.

Association of genomic features with biopsy site and tumor mutation burden

Biopsy site

We evaluated the over-representation of alterations for a given gene by calculating the following score: score=log2(%alteration of the gene at a given biopsy site/%alteration of the gene in the population). We ran a hypergeometric test to determine whether the gene is significantly over-altered for a given biopsy site. For genes related to the MMR pathway, an alteration corresponded to either a deletion or a mutation in the gene. For all other genes, we evaluate separately mutations, amplifications and deletions.

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Tumor mutation burden

We tested whether MMR-related genes, as defined in the previous section, were associated with the tumor mutation burden for each biopsy site. We ran a t-test comparing the tumor mutation burden in altered samples against wild-type samples for that purpose. The obtained p-values were subsequently corrected for multiple testing using Benjamini-Hochberg correction overall MMR-related genes.

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Competing interests.

The other authors declare no competing interests.

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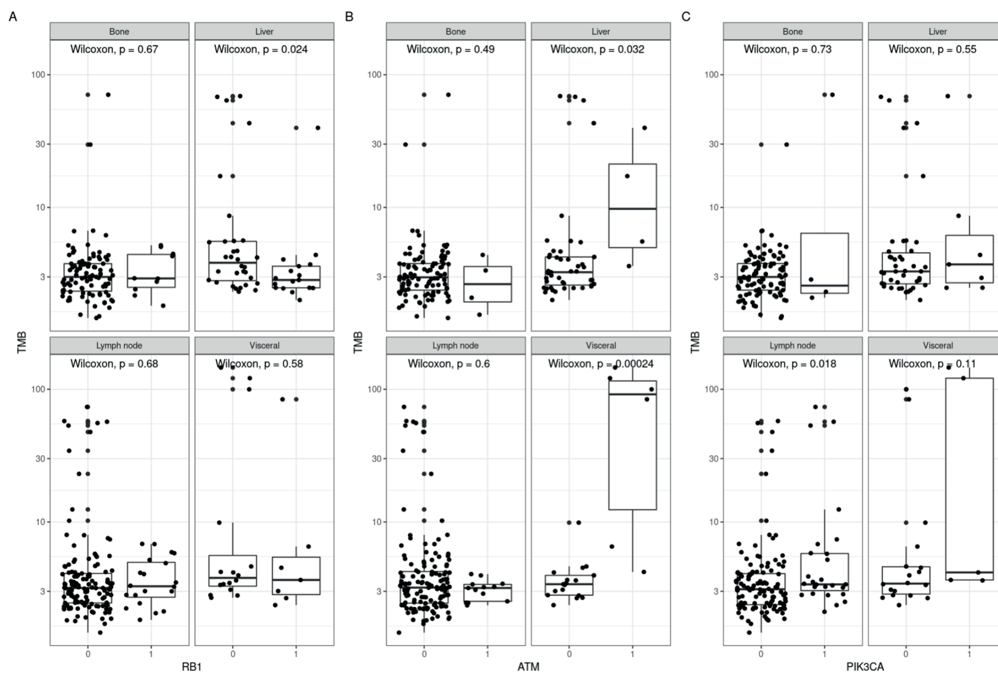
Supplementary Figures



Supplemental Figure S1: Association between TMB and *RB1*, *ATM* and *PIK3CA* gene alterations. (A-C) Boxplots showing TMB (mut/Mbp) in samples with and without *RB1, ATM or PIK3CA* gene alterations, from bone, liver, lymph node and visceral sites. Differences were assessed by a Wilcoxon test for significance.

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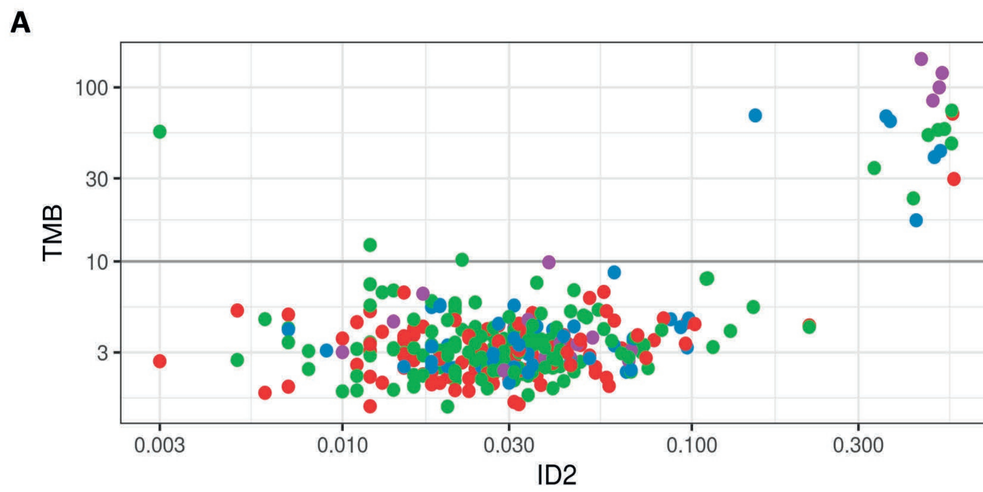

Supplemental Figure S2: High TMB samples associated with the ID2 mutational signature are enriched for liver and visceral metastases. (A) Scatter plot showing the distribution of metastatic samples based on MBT and the ID2 mutational signature association.

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Chapter 5

General discussion

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Novel molecular determinants impacting AR function and enzalutamide sensitivity with potential clinical implications

In **Chapter 2** we set out to find genes whose loss of function confer resistance to enzalutamide in prostate cancer cells. We identified transcription factor transducin-like enhancer of split 3 (TLE3) as a modulator of enzalutamide sensitivity, with loss of *TLE3* conferring drug resistance through glucocorticoid receptor (GR) function. TLE3 is a transcriptional co-repressor belonging to the transducin-like enhancer family of proteins that maintain a closed chromatin structure by recruiting repressor proteins. In a study by Stelloo et al. (2018), profiling of the endogenous AR protein interactome revealed TLE3 as a novel AR binding partner. In the same study, TLE3 was demonstrated to bind, along with FOXA1 and HOXB13, at tumor-specific AR enhancers in prostate cancer cells. Similar to FOXA1, TLE3 was shown to be present at the chromatin irrespective of androgen stimulation (Stelloo et al., 2018).
Our findings, showing that TLE3 regulates AR-mediated transcriptional output and enzalutamide resistance, are consistent with the notion that TLE3 is a core component of an AR subcomplex, which includes HOXB13 and FOXA1, that was shown to have a pivotal role in prostate cancer tumorigenesis (Pomerantz et al., 2015; Stelloo et al., 2019). Increasing evidence indicates a significant role for HOXB13 and FOXA1 in extensive reprogramming of the AR cistrome and oncogenic transformation of prostate cells (Teng et al., 2021). However, the emerging role of TLE3 in this context is incompletely understood and requires further study. Here, we present evidence implicating TLE3 as an important co-factor involved in therapy resistance and disease progression. Combined, the findings by our group and others have improved our understanding on AR function by its co-regulators, and the role of TLE3 in regulating AR function, transcriptional output, and the enzalutamide response. However, it is still unclear how exactly TLE3 loss or overexpression may affect the binding and activity of the oncogenic AR subcomplex comprising TLE3, FOXA1 and HOXB13, and how TLE3 contributes to shaping the global epigenetic landscape in prostate cancer. Genome-wide characterization of changes in epigenetic marks and transcription factor binding associated with transcriptomic changes, as a result of TLE3 loss or overexpression, may further improve our understanding on the role of TLE3 in AR activity, chromatin interactions, AR cistrome reprogramming and prostate tumorigenesis.
Transcriptomic analyses revealed that expression of a subset of AR target genes was maintained in TLE3-deficient LNCaP cells despite enzalutamide treatment. Interestingly, we identified the glucocorticoid receptor (GR) as the second-most upregulated gene in TLE3-deficient cells treated with enzalutamide. Increased GR activity has previously been implicated in therapeutic resistance to enzalutamide in pre-clinical models and

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prostate cancer patients (Arora et al., 2013; Isikbay et al., 2014; Puhr et al., 2018), and more recently in a panel of prostate cancer cell lines (Smith et al., 2020). Consistent with these findings demonstrating a role for GR in enzalutamide-resistant growth, we found that GR inhibition in *TLE3*^{KO} cells restored sensitivity to enzalutamide in our study. Importantly, we discovered that simultaneous abrogation of both TLE3 and AR function was required for this upregulation, as loss of TLE3 or AR activity alone was insufficient to induce GR expression, thus, demonstrating that AR and TLE3 are pivotal in the co-regulation of the *GR* locus in prostate cancer cells.

In an earlier study, Shah and colleagues (2017) identified a tissue-specific enhancer in prostate cancer cells responsible for *GR* regulation, revealing loss of both AR occupancy and repressive enhancer marks as critical prerequisites for GR expression. Notably, we found that TLE3 binds this specific enhancer, and revealed that concurrent loss of AR activity and TLE3 expression strongly upregulates GR expression, establishing TLE3 as a novel regulator of the *GR* locus. Combined, our data point towards TLE3 loss as one mechanism by which prostate cancer cells increase GR when exposed to AR inhibitors. However, it remains to be elucidated how decreased TLE3 expression is achieved in tumors of prostate cancer patients treated with enzalutamide. Causes may include diverse genetic and epigenetic alterations that impinge on the expression of the *TLE3* gene causing its downregulation or loss of function. In the clinic, selection of TLE3-deficient cancer cells in enzalutamide-treated patients may promote the outgrowth of resistant tumors characterized by increased GR expression. Even though our findings in pre-clinical models and patient samples reflect this, a larger sample size is needed to make this statement and reveal factors causal to alterations in TLE3 expression.

The notion that AR and GR are structurally similar, bind similar DNA response elements and recruit similar co-factors (Claessens et al., 2017; Jimenez-Panizo et al., 2019) further supports the role for GR in the context of AR substitution in enzalutamide-treated prostate cancer cells. In line with these findings, other studies using pre-clinical models for prostate cancer have shown that AR and GR have overlapping cistromes and transcriptomes (Arora et al., 2013; Sahu et al., 2013; Shah et al., 2017), allowing GR to drive enzalutamide-resistant growth by regulating expression of a subset of AR-responsive genes. Characterization of transcriptomic changes as a result of GR takeover in enzalutamide-treated prostate cancer cells may reveal novel drug targets amenable for therapeutic exploitation. Using CRISPR-Cas9 screens employing custom libraries that include GR-driven genes in enzalutamide-treated prostate cancer cells may represent an effective approach to identify such candidates. This would circumvent the need to abolish the application glucocorticoids (GCs), which are often used in the treatment of prostate cancer (Montgomery et al., 2014).

Together, the findings by our group and others indicate an intricate relationship between GR activity and the AR transcriptional program and enzalutamide response.

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Glucocorticoids, which activate GR, are often used in the treatment of prostate cancer to suppress adrenal androgen synthesis and tumor growth, reduce pain, and diminish side effects from chemotherapy (Montgomery et al., 2014). The notion that GR is able to take over AR function in prostate cancer cells treated with enzalutamide, combined with an incomplete understanding of the effects of GCs on CRPC, add a layer of complexity regarding therapeutic application of glucocorticoids in this disease setting. Analysis of a number of phase I/II clinical studies for CRPC showed significant PSA responses (20-60%) associated with GC treatment (reviewed in (Montgomery et al., 2014)). While these studies show that a significant proportion of patients experience benefit as a result of treatment with GCs, potentially, a small subset may respond adversely as a result of GR overexpression in prostate tumors. High GR expression, as a result of TLE3 loss or other mechanisms, likely represents an important determinant that dictates whether treatment with GC diminishes or exacerbates tumor growth. Future studies may build on the work described in this thesis and corroborating findings by other groups. By including the TLE3 and GR expression status in future studies investigating GCs in CRPC tumor progression, AR inhibitor response and clinical outcome, additional insight into the significance of GR in the context of enzalutamide treatment, and use of GCs, may shape patient stratification resulting in patient benefit.

In **Chapter 3** we used a kinome-centered CRISPR screen to identify enhancers of enzalutamide sensitivity in CWR-R1 prostate cancer cells, which show a moderate response to the drug. This screen identified BRAF as critical factor required for CWR-R1 cell proliferation in the presence of enzalutamide. Genetic and pharmacological inhibition confirmed that co-inhibition of AR and BRAF resulted in a strong anti-tumor effect *in vitro*. Moreover, inhibition of downstream MAPK components MEK or ERK, together with AR inhibition, recapitulated the BRAF perturbation experiments. Biochemical characterization of the MAPK pathway showed activation of the pathway in response to enzalutamide treatment. Combined, these data provided strong evidence for MAPK signaling as a critical route for cell survival in enzalutamide-treated CWR-R1 cells. Genetic characterization of the *BRAF* locus in CWR-R1 cells revealed a p.L597R mutation in the activating kinase domain of the *BRAF* gene. Furthermore, we found that two prostate cancer patients from an in-house cohort study with tumors harboring the *BRAF* p.K601E mutation showed early clinical progression despite enzalutamide treatment.
The initial response to therapy is what defines primary resistance vs acquired resistance. Primary resistance to AR antagonists in prostate cancer is observed in 10%-20% of cases, associated with clinical progression within 3 months of treatment onset. Acquired resistance is characterized by an initial response associated with tumor regression, followed by relapse of the disease (Buttigliero et al., 2015). In our study, intrinsic BRAF activity inherent to CWR-R1 cells was revealed as the key modulator of the enzalutamide

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response in these cells. Oncogenic *BRAF* alterations are a dominant mechanism underlying aberrant MAPK signaling and cell proliferation, and are characterized by hotspot mutations in the activating kinase domain of the gene (Cantwell-Dorris et al., 2011), most notably the V600E mutation found in melanoma (Davies et al., 2002) and colorectal cancer (CRC) (Tie et al., 2011). *BRAF* mutations in prostate cancer are rare, occurring in about ~2% of cases, predominantly involving the p.K601E and p.G469A hotspot mutations (Cerami et al., 2012; Gao et al., 2013). We found that CWR-R1 cells harbor the p.L597R mutation located in the activating kinase domain of *BRAF*. Expression of the BRAF p.L597R mutant in melanoma was associated with sensitivity to MEK inhibitors (Bahadoran et al., 2013; Dahlman et al., 2012). Moreover, knockdown of BRAF p.L597R in non-small cell lung cancer (NSCLC) cells demonstrated oncogenic function of this variant when compared to its wild-type counterpart (Okimoto et al., 2016). Combined, these findings illustrate the clinical relevance of the p.L597R mutation in different cancer types and sensitivity to MAPK inhibition. The more common BRAF p.K601E mutation was found present in tumors from two CRPC patients of our in-house cohort who were both unresponsive to enzalutamide treatment. Together, our *in vitro* data and clinical indicate that co-inhibition of AR and BRAF in *BRAF*-mutant prostate cancer patients could be particularly effective. However, validation of our findings implicating *BRAF* mutations in primary or acquired resistance to enzalutamide in larger cohorts is needed to confirm their causal role in AR inhibitor resistance in clinical setting.
Recent findings by Alumkal et al. (2020) further support the rationale for combined treatment of AR antagonist-sensitive prostate cancer with MAPK and AR inhibitors. In this study, transcriptional profiling of prostate cancer biopsies from 34 patients collected prior to enzalutamide treatment revealed that tumors from non-responders were characterized by an AR activity-low, stemness program. Importantly, they identified several signaling pathways whose activity was significantly enriched in non-responder vs responders. Gene set enrichment analysis (GSEA) revealed a strong enrichment for KRAS pathway activation in non-responders vs responders. Thus, patients showing a high MAPK activity prior to enzalutamide treatment showed a poor clinical response to AR inhibition (Alumkal et al., 2020), providing the rationale for co-targeting of the AR and MAPK pathway in enzalutamide-naïve tumors. Emerging evidence suggests that MAPK inhibition may also be effective in cells fully resistant to enzalutamide owing to acquired resistance. It was shown in pre-clinical models that upregulation of CXCR7 increased MAPK signaling through recruitment of ß-Arrestin 2 in enzalutamide-resistant prostate cancer cells (Li et al., 2019). Furthermore, a study investigating transcriptomic profiling of 101 mCRPC patients revealed hyperactivation of ERK1 and amplification of MAPK components in 32% of the cohort (Nickols et al., 2019). Moreover, they found that ERK1/2 phosphorylation status was associated with a poor biochemical response after radical prostatectomy. In the same study, one patient showed a potent response to the

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MEK inhibitor trametinib, after failure of multiple prior treatments including abiraterone and enzalutamide.

The presence of activating mutations in druggable kinases, including genes of the MAPK pathway, is rare in mCRPC (Robinson et al., 2015; Taylor et al., 2010). As a result, activity of MAPK inhibitors are studied poorly in clinical context. Combined, our *in vitro* and clinical data indicate that co-inhibition of AR and BRAF in *BRAF*-mutant prostate cancer patients could be particularly effective. The findings by our group and others warrant further investigation of combined inhibition of the MAPK and AR pathway at an early stage of systemic treatment of AR-driven prostate tumors showing oncogenic MAPK activation, to overcome intrinsic or acquired resistance. With the rise of genetic profiling approaches for the identification cancer mutations in clinical settings (Malone et al., 2020), the potential for combined BRAF/AR inhibition in *BRAF*-mutant prostate cancer is of particular interest for further exploration. Patients showing early detection of genetic determinants associated with activated MAPK signaling in clinical samples for prostate cancer may benefit from the combination treatment targeting the AR and MAPK pathways.

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Genetic characteristics associated with prostate cancer metastatic organotropism

In **chapter 4** we set out to identify genetic determinants associated with metastatic organotropism in prostate cancer. We analyzed molecular data collected from common CRPC metastatic sites (bone, liver, lymph node and visceral sites), using the largest WGS dataset currently reported for metastatic prostate cancer. Identification of alterations in genes or pathways associated with distinct anatomical locations may guide clinical stratification based on metastasis site, and may reveal genes amenable for therapeutic exploitation. For this study, it is important to note that it remains unclear whether genetic alterations associated with specific sites were acquired after engraftment, or were already pre-existing in clones prior to dissemination making them potentially causal in site-specific metastasis. Analysis of matched primary tumor tissue samples would be required to make any statements on this specifically.

Initial analysis of the entire data set comprising 326 samples from the same number of patients, revealed gene alterations in common drivers of prostate cancer which have been described in previously published sequencing efforts (Grasso et al., 2012; Robinson et al., 2015; Taylor et al., 2010). These studies predominantly involved primary tissue or relatively low number of metastatic samples, resulting in insufficient statistical power for comparative analyses between metastatic sites. The sample size in our study has enabled us to acquire novel insights into the distribution of these alterations across common metastatic sites.

For example, when looking into prostate cancer-relevant pathways, we found that *RB1* alterations were significantly enriched in liver metastases when compared to other sites. In mouse models of prostate cancer, *RB1*-deficiency was shown to facilitate lineage plasticity with concurrent *TP53* loss conferring resistance to antiandrogens (Ku et al., 2017). More recently, it was shown that combined inhibition of PARP and ATR resulted in significant responses in prostate cancer cells lacking RB and TP53 activity which inherently show a poor response to AR-directed therapy (Nyquist et al., 2020). Based on our data showing enrichment of *RB1* alterations in the liver, metastases of the liver lacking TP53 and RB activity may respond worse to AR inhibition, and better to PARP/ATR inhibition.

Furthermore, we found that *PIK3CA* alterations were underrepresented in bone metastases. Analysis of aggregated pathway data revealed a trend for enrichment of alterations in the PI3K pathway in lymph node compared to bone metastases, characterized by *PTEN* loss and amplifications of the catalytic subunits of PI3K. The enrichment of these alterations in the PI3K pathway suggests these metastases may be more sensitive to therapeutics targeting this pathway. However, clinical studies investigating mTOR (TORC1) inhibitors in *PTEN*-deficient prostate cancer lacked significant responses

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(Amato et al., 2008; Armstrong et al., 2010; George et al., 2020; Kruczek et al., 2013). Limited efficacy was observed for dual TORC1/2 inhibition, which was also associated with toxicity (Graham et al., 2018; Massard et al., 2017). Clinical trials investigating PI3K inhibition in prostate cancer are ongoing, or were met with disappointing results and toxicity in patients (Crumbaker et al., 2017). Lack of response to PI3K inhibitors (Hotte et al., 2019; Massard et al., 2017) may be the result of AR activation, through reciprocal feedback regulation between the PI3K and AR pathways in prostate cancer (Carver et al., 2011). Trials assessing AKT inhibition, in combination with AR inhibitors, are currently ongoing. Potentially, lymph node metastases may respond better to combined PI3K/AR inhibition compared to other anatomical sites, due to the increased frequency of alterations in the PI3K pathway.

Furthermore, we found that the tumor mutation burden (TMB) was increased in liver and visceral metastases characterized by alterations affecting mismatch repair (MMR) components *MLH1*, *MSH2* and *MSH6*. Alterations in *MLH1*, *MSH2* and *MSH6* have been found in both primary (Guedes et al., 2017; Schweizer et al., 2016) and advanced prostate cancer (Antonarakis et al., 2019; Guedes et al., 2017; Pritchard et al., 2014; Rodrigues et al., 2018; Ryan & Bose, 2019; Schweizer et al., 2016). The prevalence of alterations in these canonical MMR genes is estimated to be around 5%. A study investigating advanced prostate cancers found 12% (n=7) of 60 tumors to be hypermutated, characterized by *MSH2* and *MSH6* mutations. These biopsies included primary tissue as well as liver, bone, lymph nodes, adrenal and kidney metastases. The biological mechanism underlying the site-specific bias of MMR-deficient lesions in the liver and visceral sites remains to be elucidated. The high TMB phenotype may be acquired at metastatic sites after engraftment. Alternatively, profound genetic changes as a result of MMR-deficiency may make these tumor cells phenotypically more compatible with specific anatomical locations by modifying the stromal compartment at the pre-metastatic niche (Deng et al., 2019; Hiratsuka et al., 2006; Hoshino et al., 2015; Kaplan et al., 2005; Webber et al., 2010), or as a result of clonal selection of high TMB cells with properties favoring their engraftment at these sites (Eyles et al., 2010; Fournier et al., 2015; Jacob et al., 2015; Pani et al., 2010; Rodriguez-Torres & Allan, 2016; Sethi et al., 2011; Takeda et al., 2001).

MMR-deficiency is associated with a poor response to platinum-based chemotherapy. Although not broadly applied in prostate cancer, several studies suggest clinical relevance for this class of chemotherapeutics for the treatment of this cancer (Corn et al., 2019; Hager et al., 2016; Leal & Garcia-Perdomo, 2019; Ross et al., 2008; Schmid et al., 2020; Sternberg et al., 2009). Based on our data, future studies may emphasize on investigation of differential responses of liver and visceral metastases to platinum-based agents compared to other sites, assessing a potential role for MMR status in this context.

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Finally, high TMB and MMR-deficiency is associated with immunotherapy response, owing to the expression of neoantigens (Goodman et al., 2017). Investigation of the efficacy of monotherapy ipilumimab or pembrolizumab in unselected prostate cancer patients, was met with limited responses to these immune checkpoint inhibitors (Antonarakis et al., 2020; Beer et al., 2017; Kwon et al., 2014). However, a recent study revealed immunotherapy may benefit a small subset of prostate cancer patients with MMR-deficient tumors (Graham et al., 2020). Based on these findings and our data, patient stratification based on MMR-deficiency status and liver and visceral lesions could improve the responses to these agents.
Altogether, future studies may focus on therapy responses and clinical outcome in relation to the genetic alterations and their distribution among metastatic sites as described in chapter 4. Therapeutic responses of lesions at distinct anatomical locations may be linked to their genetic background presented in our data. Consequently, patient stratification may be shaped based on the metastasis site and genetic aberrations present in the tumor tissue, which may be identified through solid or liquid biopsies, facilitating precision medicine for prostate cancer.

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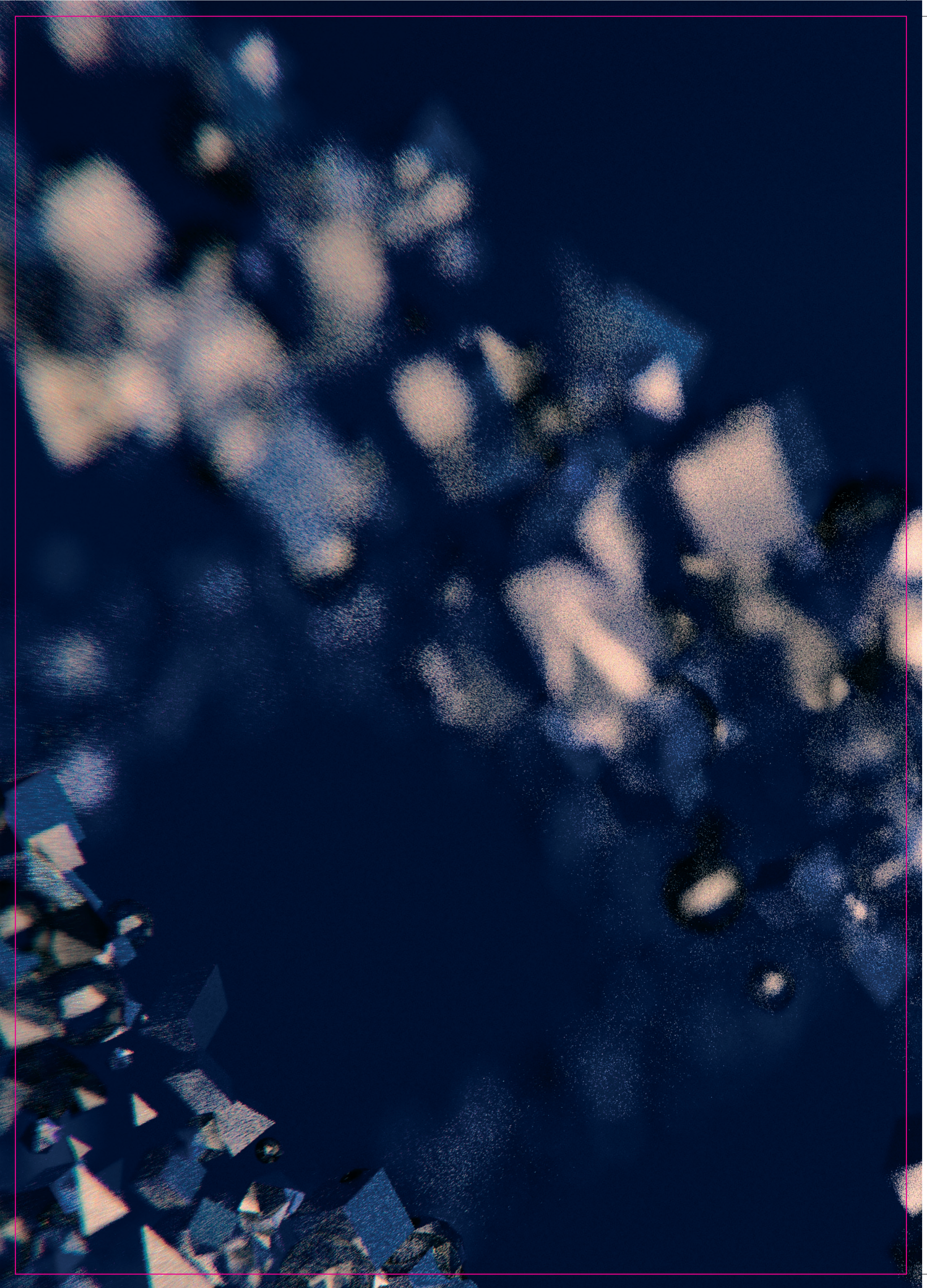

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Appendix

Nederlandse samenvatting

Summary (EN)

Curriculum vitae

Publication list

Acknowledgements

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Nederlandse samenvatting

Hoofdstuk 1 beschrijft de moleculaire basis van prostaatkanker, de huidige diagnose- en behandelmethoden, evenals de verschillende resistentiemechanismen die prostaatkankercellen ontwikkelen tegen medicijnen die gebruikt worden voor behandeling van de ziekte. Prostaatkanker is de op één na meest voorkomende vorm van kanker in mannen, met per jaar rond de 359.000 doden wereldwijd. Prostaatkanker wordt gedreven door verschillende genetische afwijkingen in genen die een belangrijke rol spelen in het reguleren van cellulaire processen zoals de celdeling en proliferatie, apoptose, DNA reparatie en hormoon biosynthese. Een belangrijke genetische afwijking die veel in prostaatkanker wordt gezien is de ontregelde expressie en activiteit van transcriptiefactoren, dit zijn de eiwitten die op het DNA de expressie van genen reguleren. Een van deze transcriptiefactoren is de androgeenreceptor (AR), welke wordt geactiveerd door binding van androgene hormonen zoals testosteron. Binding van androgene hormonen aan AR zorgt ervoor dat AR van het cytoplasma naar de celkern verplaatst, daar bindt de receptor het DNA en reguleert het de activiteit van AR target genen. Deze genen spelen een rol in de groei en differentiatie van prostaatcellen. In prostaatkanker is de expressie en activiteit van AR ontregeld, met als gevolg ontregelde celgroei. Door de belangrijke rol van AR in prostaatkanker, is het een belangrijk doelwit in de behandeling van de ziekte. Voorbeelden van veelgebruikte medicatie voor de behandeling van prostaatkanker zijn abiraterone, die de productie van androgene hormonen stillegt, en enzalutamide, een AR antagonist die de receptor bindt en daarmee inactiveert. Beide medicijnen hebben dus als doel het remmen van de AR signaalroute om zo de groei van tumorcellen te remmen. Ondanks dat AR-gerichte medicijnen aanvankelijk goed werken, is het effect vaak maar tijdelijk. Uiteindelijk worden de tumorcellen resistent tegen de AR-gerichte medicatie met als gevolg dat de prostaatkanker weer gaat groeien en deze patiënten op dat punt veelal uitbehandeld zijn. Daarom is het van belang om de moleculaire mechanismen die ten grondslag liggen aan de resistentie tegen AR remmers beter te begrijpen, om zo nieuwe therapeutische opties te ontwikkelen die de zorg voor deze patiënten verbeterd.

Hoofdstuk 2 beschrijft de ontdekking van transcriptiefactor TLE3 als een nieuwe determinant die de gevoeligheid in prostaatkankercellen tegen AR remmers moduleert. Door middel van een CRISPR-Cas9 screen in LNCaP prostaatkankercellen, vonden we dat verlies van *TLE3* ervoor zorgde dat de cellen resistent werden tegen AR remmers enzalutamide en apalutamide. Verlies van TLE3 zorgde er in deze cellen voor dat een subgroep van AR target genen vrijwel onveranderd actief bleef ondanks de aanwezigheid van AR remmers. Daarbij vonden we dat de glucocorticoïde receptor (GR) het op één na meest omhooggereguleerde gen was in enzalutamide-behandelde *TLE3* knockout (*TLE3*^{KO})

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LNCaP cellen. Analyse van ChIP-seq data liet zien dat TLE3 en AR binden op het *GR* locus, wat consistent is met de GR omhoogregulatie in *TLE3*^{KO} cellen behandeld met enzalutamide. Genetische en pharmacologische remming van GR zorgde ervoor dat *TLE3*^{KO} cellen weer gevoelig werden voor enzalutamide. Deze bevindingen zijn in lijn met de binding van GR bij de TLE3/AR-gedeelde genen die actief blijven in enzalutamide-behandelde *TLE3*^{KO} cellen. Analyse van TLE3 en GR expressie in patienten samples reflecteren de *in vitro* bevindingen. Door het relatief kleine aantal klinische samples dient de rol van TLE3 en GR in de resistentie tegen AR remmers verder onderzocht te worden in grotere cohorten om zo de klinische significantie ervan te bepalen.

In **hoofdstuk 3** hebben we een kinome-georiënteerde CRISPR-Cas9 screen uitgevoerd in CWR-R1 prostaatkankercellen, om te onderzoeken welke kinases mogelijk een rol spelen in de gevoeligheid voor AR remmers. We vonden dat enzalutamide resistentie werd gedreven door activatie van de BRAF signaalroute in deze cellen. BRAF is een component van de MAPK signaalroute die een belangrijke rol speelt bij celgroei. Pharmacologische inhibitie van zowel de MAPK als de AR signaalroute zorgde voor een sterke remming in de groei van CWR-R1 cellen, terwijl gebruik van elke remmer afzonderlijk geen effect had. Groei-experimenten met CWR-R1 *BRAF*^{KO} cellen lieten vergelijkbare resultaten zien. Hierbij had verlies van BRAF expressie als gevolg van CRISPR-Cas9-gemedieerde editing geen gevolgen voor celgroei, maar toevoeging van enzalutamide zorgde voor een sterke remming in proliferatie. Karakterisatie van het *BRAF* gen liet een oncogene mutatie zien in het activerende kinase domein van het eiwit, de p.L597R mutatie. De associatie tussen mutatie van het activerende kinase domein van BRAF en de enzalutamide response vonden we ook in patienten samples. Twee patienten met de p.K601E mutatie lieten een slechte response op enzalutamide zien. Gezamenlijk wijzen deze data naar een rol voor BRAF activatie, als gevolg van mutaties in het kinase domein, als een resistentiemechanisme voor enzalutamide. De resultaten in deze studie wijzen op het belang om de klinische relevantie van MAPK en AR remming in MAPK-geactiveerde prostaatkanker verder te onderzoeken.

In **hoofdstuk 4** onderzochten we prostaatkankermetastasen afkomstig van verschillende organen, met als doel het identificeren van genetische eigenschappen die mogelijk geassoccieerd zijn met orgaanspecifieke metastasering. Metastasering van tumoren naar specieke weefsels gebeurt volgens een niet-stochastisch process genaamd "metastatisch organotropisme". Om dit te onderzoeken in prostaatkanker, hebben we 326 biopten afkomstig van lymfeklieren, lever, bot, en viscerale prostaatkankermetastasen genetisch gekarakteriseerd. Daarbij hebben we gekeken naar mutaties, deleties en amplificaties in deze samples. We vonden dat genetische alteraties in *RB1* en *PIK3CA* verrijkt waren in metastasen van respectievelijk de lymfeklieren en de lever. Wanneer

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we keken naar de geagreggeerde signaalroute data, vonden we een trend voor verrijking van alteraties in de PI3K en DNA reparatie signaalroutes in lymfekliermetastasen vergeleken met samples afkomstig van het bot. Analyse van de tumor mutation burden (TMB), wat een maat is voor het totale aantal mutaties dat wordt gevonden in het genoom van de tumor, liet zien dat deze hoger was in viscerale en lever metastasen, vergeleken met bot en lymfekliermetastasen. Bovendien lieten de samples met hoog TMB verrijking zien voor een mismatch-repair (MMR) deficiëntie signatuur. In lijn met deze bevindingen, zagen we dat er een overrepresentatie was van alteraties in MMR genen in viscerale en levermetastasen met hoog TMB. Levermetastasen met hoog TMB werden gekenmerkt door alteraties in *MSH6*, *MLH1* en *POLD3*, terwijl viscerale metastasen met hoog TMB gekarakteriseerd werden door alteraties in *MSH2* en *POLD1*. Samenvattend, laten deze data zien dat in prostaatkankermetastasen afkomstig van verschillende organen er een differentiele verrijking is van genetische alteraties in prostaatkanker-relevante oncogenen, evenals de TMB en alteraties in MMR genen. Deze eigenschappen die de metastatische organotropisme in prostaatkanker karakteriseren hebben mogelijk invloed op het ziekteverloop en therapie response.

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Summary (EN)

In **Chapter 1** an overview is given describing the molecular basis of prostate cancer, current diagnosis and treatment approaches for the disease, and resistance mechanisms to commonly used therapeutics. Prostate cancer is the second-most common cancer diagnosed in men, responsible for over 359.000 deaths worldwide each year. Prostate tumorigenesis is driven by various genetic alterations affecting genes implicated in various cellular processes including cell division and proliferation, apoptosis, DNA repair and hormone biosynthesis. Importantly, prostate cancer is characterized by the aberrant expression and activity of several of transcription factors, most notably the androgen receptor (AR). The AR signaling pathway is stimulated in response to androgens which bind the receptor, facilitating AR translocation to the nucleus where it binds the DNA and regulates target genes that have a central role in cell differentiation, growth and survival. In prostate cancer, aberrant AR function leads to the deregulation of AR target gene expression, driving prostate tumorigenesis. Due to its role as a major driver in prostate cancer, treatment of the disease centers around controlling the AR therapeutically. Examples of cornerstone drugs used for the treatment of prostate cancer are abiraterone, which lowers the level of androgens and androgen precursors in the serum of patients, and enzalutamide, which functions through antagonistic binding of AR shutting down the pathway. Even though AR-directed therapies are effective initially in the majority of cases, durable responses are limited and resistance to these drugs inevitably occurs, resulting in patient death. Therefore, there is a pressing need to better understand therapeutic resistance to AR inhibitors and identify novel treatment avenues to improve prostate cancer patient care.

Chapter 2 describes the discovery of TLE3 as a novel regulator of enzalutamide and apalutamide resistance using a genome-wide CRISPR-Cas9 resistance screen in LNCaP cells. At the molecular level, *TLE3* loss rescued expression of a subset of AR target genes in enzalutamide-treated cells. The second-most upregulated gene in enzalutamide-treated *TLE3*^{KO} cells was the glucocorticoid receptor (GR), which was previously shown to substitute AR in prostate cancer cells thereby conferring enzalutamide resistance. We found that TLE3 and AR bind at the *GR* locus, consistent with upregulation of GR in this context. Genetic and pharmacological perturbation of GR rescued the resistance phenotype. Moreover, GR binding at TLE3/AR-shared genes further supports the role of GR in enzalutamide resistance in *TLE3*^{KO} cells. Analysis of patient samples revealed an association between TLE3 and GR expression in prostate tumors that are in line with our *in vitro* findings, of which the clinical relevance is yet to be established. Combined, we revealed TLE3 as a novel modulator of AR inhibitor sensitivity and regula-

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tor of the *GR* locus dictating GR expression in enzalutamide-treated *TLE3*^{KO} cells. Further validation of our findings in a clinical setting is need to establish the clinical relevance of GR-mediated AR inhibitor resistance in TLE3-deficient prostate cancer cells.

Chapter 3 reveals aberrant MAPK signaling as a determinant of enzalutamide sensitivity in CWR-R1 prostate cancer cells. Using a kinome-centered CRISPR-Cas9 screen, we identified BRAF as a critical node required for cell growth in enzalutamide-treated CWR-R1 cells. Inhibition of downstream MAPK components MEK or ERK in conjunction with enzalutamide yielded similar results, showing strong synergistic inhibition of cell proliferation. Characterization of the *BRAF* gene revealed a mutation in the activating kinase domain of BRAF. The lack of response to enzalutamide in two patients harboring mutations in the activating kinase domain of BRAF is consistent with our findings in CWR-R1 cells *in vitro*. Combined, our findings suggest that co-targeting of the AR and MAPK pathway may be effective in patients with an activated MAPK pathway, particularly those harboring tumors with oncogenic *BRAF* mutations. Together our findings warrant further investigation of the AR inhibitor response and co-inhibition of AR and MAPK signaling in *BRAF*-mutant prostate tumors in a clinical setting.

In **Chapter 4** we explore molecular data with the aim to reveal genetic determinants associated with metastatic organotropism in prostate cancer. Metastatic organotropism is the non-random process of site-specific metastasis of tumor cells. The association between metastatic site and patient survival emphasizes the need to better understand metastatic organotropism in prostate cancer. To this end, we set out to characterize genetic features associated with metastasis site through molecular profiling of 326 prostate cancer metastases, hitherto the largest dataset for metastatic prostate tumor tissue. We found *RB1* and *PIK3CA* alterations to be enriched in metastases of the lymph nodes and liver respectively when compared to other sites. Aggregated pathway alteration data showed a trend for enrichment of PI3K and DNA repair pathway alterations in lymph node compared to bone metastases. Furthermore, analysis of TMB revealed it was increased in visceral and liver metastases, which was associated with an MMR-deficiency signature. In line with these findings, a significant proportion of high-TMB liver metastases showed alterations in *MSH6*, *MLH1* and *POLD3*, while high-TMB visceral metastases were characterized by *MSH2* and *POLD1* alterations. Together, our data show differential enrichment of TMB/MMR-deficiency, and alterations affecting prostate cancer-related oncogenic drivers, at distinct metastatic sites, which may potentially impact therapy response and disease progression.

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Curriculum vitae

Sander Palit was born on the 4th of September 1987, in Oss, The Netherlands. After obtaining his Bachelor of Applied Sciences degree in Biochemistry and Molecular Biology at the Hogeschool Arnhem and Nijmegen (HAN), he started his Master in Medical Biology at Radboud University focusing on molecular biology in cancer. During his first internship as a Master student at the Pediatric Oncology lab of Prof. F. van Leeuwen, he studied the role of tumor suppressor BTG1 in the cell stress response in B-cell acute lymphoblastic leukemia. Following his first internship, he went on to do his second internship at the Netherlands Cancer Institute (NKI), where he studied the role of DOT1L in oncogenic transformation of T-cells using mouse T-cell lymphoma models. In June 2015 he received his Master's degree and directly started his PhD training in the lab of Dr. Michiel van der Heijden. For his research, he used functional genomic approaches to identify and study genetic determinants that have a central role in prostate cancer biology and therapy response, the result of which is described in this thesis.

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