Regulation of the glucocorticoid receptor in prostate cancer disease progression and resistance to anti-androgen therapy

by

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Abstract

Acquired resistance to targeted cancer therapies is most commonly caused by fixed genomic alterations in the drug target, such as mutations or gene amplifications, that restore oncogenic growth despite continued treatment. In prostate cancer, resistance to the anti-androgen enzalutamide (Enz) can occur through bypass of androgen receptor (AR) blockade by the glucocorticoid receptor (GR). In contrast to fixed genomic alterations, we show that GR-mediated anti-androgen resistance is adaptive and reversible due to regulation of GR expression by a tissue-specific enhancer present in normal prostate cells. GR expression is silenced in prostate cancer by a combination of repressive AR binding at this enhancer and EZH2-mediated repression at the GR locus, but is restored in advanced prostate cancers upon reversion of both repressive signals. Remarkably, BET bromodomain inhibition resensitizes drug-resistant tumors to Enz by selectively impairing the GR signaling axis, but not the AR signaling axis, via this enhancer. Thus, prostate cancers can develop resistance to AR targeted therapy by reactivating a normally silenced enhancer, which can be reversed with remarkable selectivity by a BET inhibitor. In addition to revealing the underlying molecular mechanism behind drug resistance, these data suggest that inhibitors of broadly active chromatin-readers could have utility in nuanced clinical contexts of acquired drug resistance with a more favorable therapeutic index.

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List of abbreviations

Abi	Abiraterone
ACTH	Adrenocorticotropic hormone
ADT	Androgen deprivation therapy
AR	Androgen receptor
ARE	Androgen response element
BET	Bromodomain and Extra-Terminal
BETi	BET inhibitor/inhibition
Chem-seq	Chemical-pulldown sequencing
ChIP-seq	Chromatin immunoprecipitation sequencing
CRPC	Castration resistant prostate cancer
Dex	Dexamethasone
DHT	Dihydrotestosterone
Enz	Enzalutamide
ER	Estrogen receptor
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
GSEA	Gene set enrichment analysis
GSI	Gamma-secretase inhibitor
HSP90	Heat shock protein 90
LBD	Ligand binding domain
LH	Luteinizing hormone

LNAR'	LnCAP-AR cell lines (prime denoting passage in vivo)
LREX'	LNAR resistant to Enzalutamide xenograft cell line (prime denoting passage in vivo)
NEPC	Neuroendocrine prostate cancer
NR3C1	Nuclear receptor subfamily 3 group C member 1 (GR gene name)
PR	Progesterone receptor
PRC2	Polycomb repressive complex 2
PSA	Prostate specific antigen
sgRNA	Synthetic guide RNA

1. Introduction

1.1 Prostate cancer treatment: targeting the androgen signaling axis

Prostate cancer is currently the third leading cause of cancer related deaths in the United States, and it is estimated that over 160,000 new cases will be diagnosed every year (Haas et al. 2008). In early stages of the disease, where the cancer is still localized to the prostate gland, treatment via surgery (prostatectomy) and radiation therapy are often curable. With earlier detection methods, such as non-invasive screening of serum prostate-specifc antigen (PSA) levels, mortality has decreased significantly due to management and treatment of prostate cancer at earlier stages of the disease (Buzzoni et al. 2015). However, when prostate cancer metastasizes, often times to the bone causing painful osteoblastic lesions, patient mortality increases drastically with a five-year patient survival rate of below 30%.

Common treatment options for metastatic prostate cancer involve androgen deprivation therapy (ADT), which is dependent on the fact that prostate cancer cells require androgens, primarily in the form of dihydrotestosterone (DHT), for their continued growth and survival. The androgen receptor (AR) is the primary steroid hormone receptor and transcription factor through which DHT binds to and functions in prostate tissue (Zhou et al. 1995). In the absence of DHT ligand, AR is primarily localized in the cytoplasm where it associates with heat shock protein 90 (HSP90) as well as other chaperones which modulate its nuclear localization and transcriptional ability (Azad et al. 2015b; Fang et al. 1996). Upon binding DHT, AR undergoes a conformational change, dimerizes, and becomes phosphorylated. The AR homodimer

dissociates with HSP90, and HSP27 helps traffic the ligand bound steroid receptor to the nucleus (Zoubeidi et al. 2007). With the help of co-regulators, AR binds to androgen response elements (AREs) at promoters and enhancers to transcriptionally regulate key genes which are necessary for the continued growth and survival of the prostate cancer cells (Dehm and Tindall 2006).

There are two main strategies to target the androgen signal axis in the treatment of advanced prostate cancer: the first is to prevent androgen synthesis at the source; and the second is to block AR activity in the target tissue. Synthesis of androgens is largely under the regulation of the hypothalamic–pituitary–testicular hormone network. Drugs such as gonadotropin-releasing hormone (GnRH) agonists and antagonists effectively disrupt this network by preventing hypothalamus-produced GnRH from acting on the pituitary gland, which subsequently prevents luteinizing hormone (LH) from activating androgen production in the testes. Combined androgen blockade incorporates the use of AR antagonists, termed anti-androgens, which effectively prevent DHT and other androgens from binding to AR. This further impedes androgen signaling in the target tissue by preventing AR from activating its downstream target genes which are necessary for prostate cancer growth. Together, these two strategies form the backbone of ADT in the first-line treatment of metastatic prostate cancer (Watson et al. 2015).

Despite initial responses to ADT, patients invariably relapse to a lethal stage of the disease termed castration-resistant prostate cancer (CRPC) where the cancer will continue to grow despite ongoing targeted therapies. This often presents in the clinical setting as rising levels of serum PSA, new metastatic lesions, and increase in tumor size (Lonergan and Tindall 2011). However, the majority of CRPC cases are still dependent

on AR-mediated signaling for their continued growth, and the residual levels of androgens that remain after first-line ADT are crucial in this disease progression. For example, over 30% of CRPC patients harbor genomic amplifications of the AR gene locus that are not present prior to ADT (Visakorpi et al. 1995). Furthermore, it was shown that AR overexpression was both necessary and sufficient to cause resistance to ADT by sensitizing AR to lower levels of ligand, and also by causing first-generation anti-androgens such as bicalutamide to become agonists in this setting (Chen et al. 2004).

Given the continued dependence of CRPC on androgen-mediated growth, newer drugs to more effectively target the androgen signaling axis were recently discovered. The second-generation anti-androgen enzalutamide (Enz) is a much more potent inhibitor of AR. Even in the setting of AR amplification, Enz acts as an effective AR antagonist, preventing DHT from binding as a ligand, reducing the efficiency of AR nuclear translocation, and impairing both DNA binding and co-regulator recruitment (Tran et al. 2009). The drug abiraterone acetate (Abi) was discovered as a CYP17A1 inhibitor, an enzyme which is involved in de novo steroidogeneis by producing DHT precursors in the adrenal glands, an important source of residual androgens in CRPC (Attard et al. 2005). Based on clinical trial results showing a significant improvement in overall patient survival, both Enz and Abi were approved for the treatment of CRPC in chemotherapy-naive and chemotherapy-refractory patients (Scher et al. 2012; de Bono et al. 2011). Despite improvements in survival, acquired resistance to Enz and Abi remains a major clinical challenge, and understanding how resistance arises to these second-generation AR-targeted therapies is imperative in improving treatment strategies for advanced cases of prostate cancer.

1.2 The prostate gland: a cellular hierarchy

The prostate gland is an important part of the male reproductive system that functions in producing major constituents of seminal fluid necessary for spermatozoid survival. The prostate itself consists of glandular epithelial cells embedded in a fibromuscular stroma, and is comprised of two distinct layers, the secretory luminal cells and the basal cells that surround them. Both of these cell types play an important role in prostate development and function, and can be distinguished histologically by both their appearance and the presence of specific cell markers. The neuroendocrine cells are another epithelial cell type found in the prostate. They do not constitute a distinct layer within the prostate gland, but are sparsely scattered throughout the basal and luminal cells.

The basal cells are androgen-independent in nature and are distinguished by the presence of specific cytokeratin (CK) markers, including CK5 and CK14. The cell surface marker CD44, the anti-apoptotic Bcl-2, and the p53 homologue p63 are also commonly used markers to characterize them. Histologically, these cells surround the luminal cells in the prostate gland, and function as the main proliferative compartment of the prostate gland during development (Long et al. 2005; Wang et al. 2001; Bonkhoff et al. 1994; van Leenders et al. 2000).

The luminal cells are the secretory cells of the prostate gland, and comprise the majority of cells in prostate cancer. They are dependent on androgen signaling for growth and survival and express high levels of the androgen receptor (AR) (Wang et al. 2001; Long et al. 2005; Sherwood et al. 1990; Okada et al. 1992). Upon androgen withdrawal, these cells undergo apoptosis and die, which is the premise behind hormone therapy for

the treatment of prostate cancer (Long et al. 2005). Luminal cells are characterized by expressing CK8 and 18, and the cell surface marker CD57. These cells function in secreting important constituents of seminal fluid, including prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and kallikrein-2 (KLK2), which are expressed via direct AR transcriptional activity (Bennett et al. 2010; Wernert et al. 1986).

Neuroendocrine cells are the other type of epithelial cell present in the prostate gland. This cell type is relatively rare in the prostate, found sparsely scattered between basal and luminal cells, and is not specific to a distinct layer. They are characterized by the expression of chromogranin A and synaptophysin. Neuroendocrine cells are androgen-independent, though their function in the prostate is still not fully understood. Some evidence suggests that they may play a role in the growth and differentiation of the developing prostate gland (Long et al. 2005; Noordzij et al. 1995; Hirano et al. 2004).

Serial androgen withdrawal and subsequent re-administration experiments in rat prostates result in the regeneration of a fully functional prostate gland (Huggins and Hodges 2002; Kyprianou and Isaacs 1988; DeKlerk and Coffey 1978). Experiments like these have pointed towards the existence of a prostate stem cell or progenitor cells that are able to reconstitute all cell types of the prostate gland over multiple regenerations. Identifying and characterizing these cells, however, has proven difficult.

Androgen withdrawal leads to apoptosis of the majority of the luminal cells, while the basal cells remain relatively unharmed (DeKlerk and Coffey 1978; Huggins and Hodges 2002; Kyprianou and Isaacs 1988) . This, and transplantation experiments of basal cells (Leong et al. 2008), provided evidence that in the adult prostate, there is a multipotent progenitor cell in the basal compartment that can give rise to both basal and

luminal cells. However, recent lineage tracing experiments have shown that adult prostate basal and luminal cells are independently self-sustained lineages in vivo (Choi et al. 2012). As such, there is still debate as to how the prostate gland is maintained and self-renewed upon androgen regeneration experiments.

Studies of developing and intact prostate glands have led to the discovery of so-called “intermediate cells” that express markers and properties of both basal and luminal cells (Long et al. 2005; Ousset et al. 2012; van Leenders et al. 2000; van Leenders et al. 2001; Xue et al. 1998; Verhagen et al. 1988). In particular, these intermediate cells express both basal-CK5 and luminal-CK18 markers, but are absent in p63 and CK14. Research has provided evidence that this intermediate cell type marks a transit-amplifying cell that ultimately differentiates into a luminal cell, and is androgen-independent (Ousset et al. 2012; Long et al. 2005). These intermediate cells can be found in both the luminal and basal layers of the prostate gland (van Leenders et al. 2000; Wang et al. 2001). They are highly expressed in developing prostate tissue but quite rare in a fully-developed intact prostate gland. Some research has also suggested that this intermediate cell type may play an important role in resistance to current hormone therapies used to treat prostate cancer, though this has never been definitively shown (Long et al. 2005; Wang et al. 2012)

1.3 Mechanisms of resistance to AR therapies

Resistance to AR-targeted therapies can be classified in to three general categories: restoration of AR-signaling; complete AR independence; and AR-bypass signaling (Watson et al. 2015).

As mentioned earlier, AR gene amplification is one mechanism by which prostate cancers can restore AR-signaling in the presence of ADT. Mutations in the AR ligand binding domain (LBD) are another mechanism by which this can be accomplished. These mutations can turn AR-antagonists in to agonists, thereby activating AR-signaling in the presence of specific anti-androgens. For example, certain hot-spot mutations in the LBD (L702H, W742C, H875Y and T878A), which are present in ~20% of CRPC cases, can cause specific first-generation anti-androgens to act as agonists, including flutamide, nilutamide, and bicalutamide (Beltran et al. 2013; Robinson et al. 2015b; Tan et al. 1997; Taplin et al. 1999; Hara et al. 2003). The presence of these mutations in patient tumors can often cause what is termed 'anti-androgen withdrawal syndrome', whereby tumors which once responded to therapy can regress upon termination of anti-androgen treatment (Scher and Kelly 1993). Activating AR LBD mutations can also effect second-generation anti-androgens, such as Enz. The F877L mutation can cause Enz to act as an AR agonist, and has been reported in a small percentage of patients via circulating cell-free DNA analysis (Balbas et al. 2013; Azad et al. 2015a). Interestingly, certain LBD hotspot mutations, including L702H and T878A, can arise in prostate tumors which have not previously been exposed to anti-androgens, suggesting an alternative selective pressure for their incidence. Recent studies have shown that these mutations can cause ligand promiscuity, whereby AR can be activated by other steroid hormones, including

progesterone and glucocorticoids (Veldscholte et al. 1990; Zhao et al. 2000) . As such, the L702H mutation, which allows AR to be activated by glucocorticoids, has been observed with increasing frequency in patients who are treated with prednisone (a synthetic glucocorticoid), which is often prescribed to ameliorate the side effects of certain ADT treatments (Carreira et al. 2014).

The presence of AR splice variants in prostate tumors has also been implicated with CRPC and resistance to Enz/Abi (Ware et al. 2014; Nakazawa et al. 2014). One particular variant, AR-V7, has been the focus of many studies as it lacks the LBD, but retains the amino-terminal transactivation and DNA-binding domains, and has constitutive nuclear localization and transcriptional activity in the absence of ligand (Guo et al. 2009). However, the precise mechanism of AR-V7 activity is still largely unknown, as under certain cellular contexts, AR-V7 expression is neither necessary nor sufficient to drive ADT resistance (Li et al. 2013; Watson et al. 2010). Nevertheless, AR-V7 expression in circulating tumor cells is a useful biomarker for those patients who will not respond to Enz/Abi, and alternatively, may benefit from taxane-based chemotherapy (Antonarakis et al. 2014; Scher et al. 2016).

Another mechanism by which prostate cancers can become resistant to AR-directed therapies is by transitioning to a completely AR-independent state of the disease. Prostate cancers themselves are very heterogeneous in nature, and not all cells in the tumor will share distinct pathological features. For example, AR staining can be quite diverse, both at an intra- and inter-tumoral level, and over 8% of patients can show very little to no AR-staining in their tumor samples (Crnalic et al. 2010). Often times, these AR-negative tumors can display features of neuroendocrine like prostate cancer (NEPC),

including expression of chromogranin A and synaptophysin markers, as mentioned previously, or histologically resemble small-cell carcinoma, both AR-independent forms of the disease (Palmgren et al. 2007; Epstein et al. 2014). NEPC often carries mutations or deletions in TP53 and RB1 genes, and with the advent of more effective drugs to target the AR-pathway such and Enz/Abi, these mutations are becoming increasingly prevalent in the landscape of genomic alterations in resistant CRPC (Tan et al. 2014; Beltran et al. 2011; Robinson et al. 2015b). In fact, loss of TP53 and RB1 was shown to promote lineage-plasticity in prostate cancer cells to a more basal and neuroendocrine-like phenotype, which can ultimately drive anti-androgen resistance (Mu et al. 2017; Ku et al. 2017).

The last category of resistance to AR-targeted therapies is AR-bypass signaling. This can the accomplished via the up-regulation of the glucocorticoid receptor (GR), which can effectively bypass the need for direct AR-signaling and drive the expression of a subset of AR-target genes. The remainder of this thesis will focus on the importance of GR in anti-androgen resistance, its regulation in disease progression, and a subsequent approach to specifically target GR expression in the CRPC setting.

2. Regulation of the glucocorticoid receptor in prostate cancer

2.1 Introduction: The role of GR in Enz-resistance

To study resistance mechanisms to Enz in a pre-clinical setting, a human LNCaP xenograft model with an AR overexpression construct (LNAR) was utilized (Chen et al. 2004). LNAR cells were injected subcutaneously in to castrated mice and treated with Enz once tumors reached a sufficient size. Not surprisingly, tumors initially regressed upon Enz treatment; however, after several months, the tumors began to grow again despite ongoing Enz exposure. Transcriptome analysis from the resultant resistant tumors revealed a significant upregulation of the glucocorticoid receptor (GR) in the majority of samples when compared to the control sensitive tumors (Arora et al. 2013).

The fact that GR was highly upregulated in Enz-resistant tumors was of particular interest, as it has previously been shown that GR and AR have similar DNA consensus sites and share a large number of binding sites across the genome (Sahu et al. 2013). This led to the hypothesis that GR could bypass the need for direct AR signaling under androgen-deprived conditions by binding to and driving the expression of a subset of AR target genes. Consistent with this hypothesis, GR was shown to bind over 50% of AR target genes in the LNAR resistant model, and this led to activation of these genes in the Enz-resistant tumors. GR was also shown to be necessary for Enz-resistance, as knockdown of GR expression could resensitize these tumors to Enz. Furthermore, analysis of human tumor samples showed a significant upregulation of GR expression in patients who responded poorly to Enz (Isikbay et al. 2014; Arora et al. 2013).

Together, this data demonstrates the importance of GR in driving Enz-resistance both in a pre-clinical model and in the clinical setting. Nevertheless, how GR expression is regulated in prostate cancer disease progression is still largely unknown. In the first section of this thesis, we will address this issue and determine how GR expression is modulated in prostate cancer, and the molecular mechanisms behind its transcriptional regulation.

2.2 Results

2.2.1 A tissue-specific enhancer regulates GR expression

As mentioned previously, to study acquired resistance mechanisms to ADT, we employed the AR-dependent LNAR mouse xenograft model, previously used to demonstrate the activity of Enz (Tran et al. 2009), and we identified GR upregulation as a driver of Enz resistance (Arora et al. 2013). LNAR cells were injected subcutaneously into castrate mice and treated with either vehicle or Enz for an extended period of time until resistant tumors formed. Vehicle-treated (LNAR') and Enz-resistant (LREX') tumors were then adapted back into in vitro culture conditions for further molecular characterization (Fig 2.1A). GR upregulation was found in 8 of 13 independent Enz-resistant tumor clones. GR expression in LREX' cells was dynamic and could be toggled on and off based on Enz exposure, consistent with an AR-mediated negative feedback (Fig 2.1A). Conversely, Enz treatment did not result in significant GR upregulation in Enz-sensitive LNAR' cells or in GR-negative resistant clones (Fig 2.1B), suggesting a second level of GR regulation beyond AR inhibition. The importance of GR as a

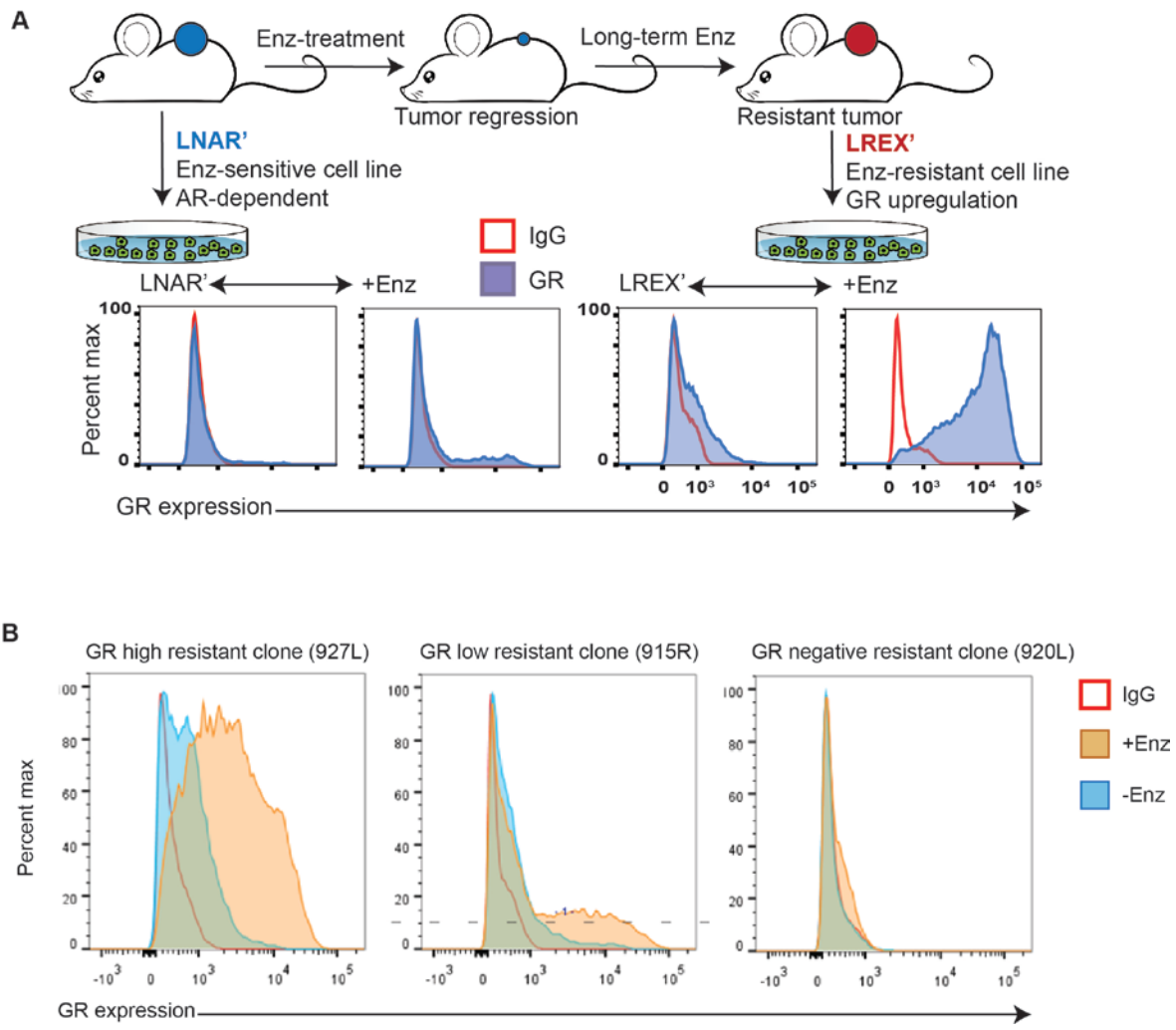

Fig 2.1 - The LNAR'/LREX' prostate cancer xenograft model to study Enz resistance

A) Top - LNAR' Enz-sensitive and LREX' Enz-resistant cell lines were derived from in vivo xenograft experiments.). LNAR' (previously known as CS1 (Arora et al. 2013)) and LREX' cell lines were derived from in vivo xenograft experiments, and adapted back into in vitro culture conditions with or without 1uM Enz. Bottom - Flow cytometry on LNAR' and LREX' cell lines for GR expression in the presence and absence of Enz (1uM).

B) Flow cytometry of GR expression in different Enz-resistant clones in the presence or absence of Enz exposure: GR-high (927L) and GR-low (915R) clones show consistent GR-dependent expression on Enz-exposure; GR-negative (920L) resistant clone shows no GR induction.

resistance mechanism was also evident in the CRPC organoid line (MSK-PCa2) (Gao et al. 2014), where dexamethasone (Dex) treatment partially restored growth and rescued AR target gene expression in the presence of Enz (Fig 2.2).

Whole exome sequencing of LNAR' and LREX' cells did not reveal any gene-coding mutations associated with Enz resistance; therefore, we considered epigenetic mechanisms. Previous studies of GR transcriptional regulation have largely focused on the proximal promoter, which is the primary mechanism of GR regulation in most tissue types (Breslin et al. 2001; McGowan et al. 2009; Nobukuni et al. 1995; Nunez and Vedeckis 2002). To examine GR regulation in the context of this prostate model, we used chromatin-immunoprecipitation followed by sequencing (ChIP-seq) to survey the chromatin landscape for the H3K4me3, H3K4me1 and H3K27ac histone modifications, then examined these marks across the GR gene (*NR3C1*) locus (Fig 2.3A). This analysis identified a clear enhancer (revealed by the H3K4me1 track) (Rada-Iglesias et al. 2011; Creyghton et al. 2010) in LNAR' and LREX' cells, regardless of Enz treatment. Consistent with the high level of GR mRNA expression in LREX' cells treated with Enz, this enhancer and the GR promoter both had clearly defined active chromatin marks (H3K27ac and H3K4me3, respectively). Conversely, the lack of these active marks in LNAR' cells signified a poised GR enhancer state.

Curiously, this enhancer has not been reported in earlier studies of GR transcriptional control, largely conducted in other tissue types. To investigate the role of the enhancer in regulating GR expression in this prostate cancer model, we designed two independent CRISPR guide RNA pairs (sgRNA) flanking the ~4.7kb enhancer to excise the entire region (Fig 2.3B). Anticipating that LREX' cells treated with Enz may not

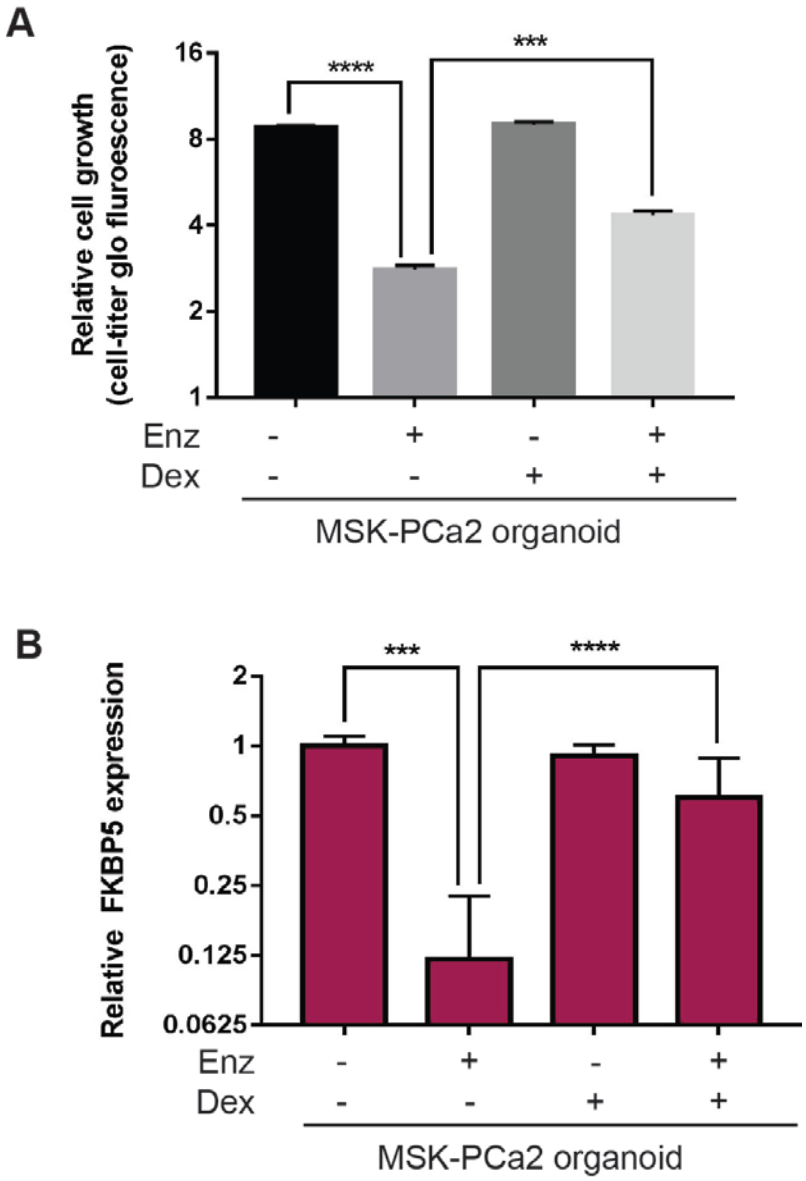

Fig 2.2 - GR activation drives Enz resistance in prostate cancer organoids

A) MSK-PCa2 organoid lines treated with Enz (1uM) or Dex (100nM) over 4 days. Relative organoid growth as measured by Cell-Titer Glo assay. Organoid lines were derived from human normal luminal prostate tissue (CD26+ cells) or human advanced prostate cancer tissue (MSK-PCa2 organoid line), and maintained in 3D culture conditions, as previously described.

B) RT-qPCR for FKBP5 as a readout of AR/GR target gene activation, normalized to veh treated samples.

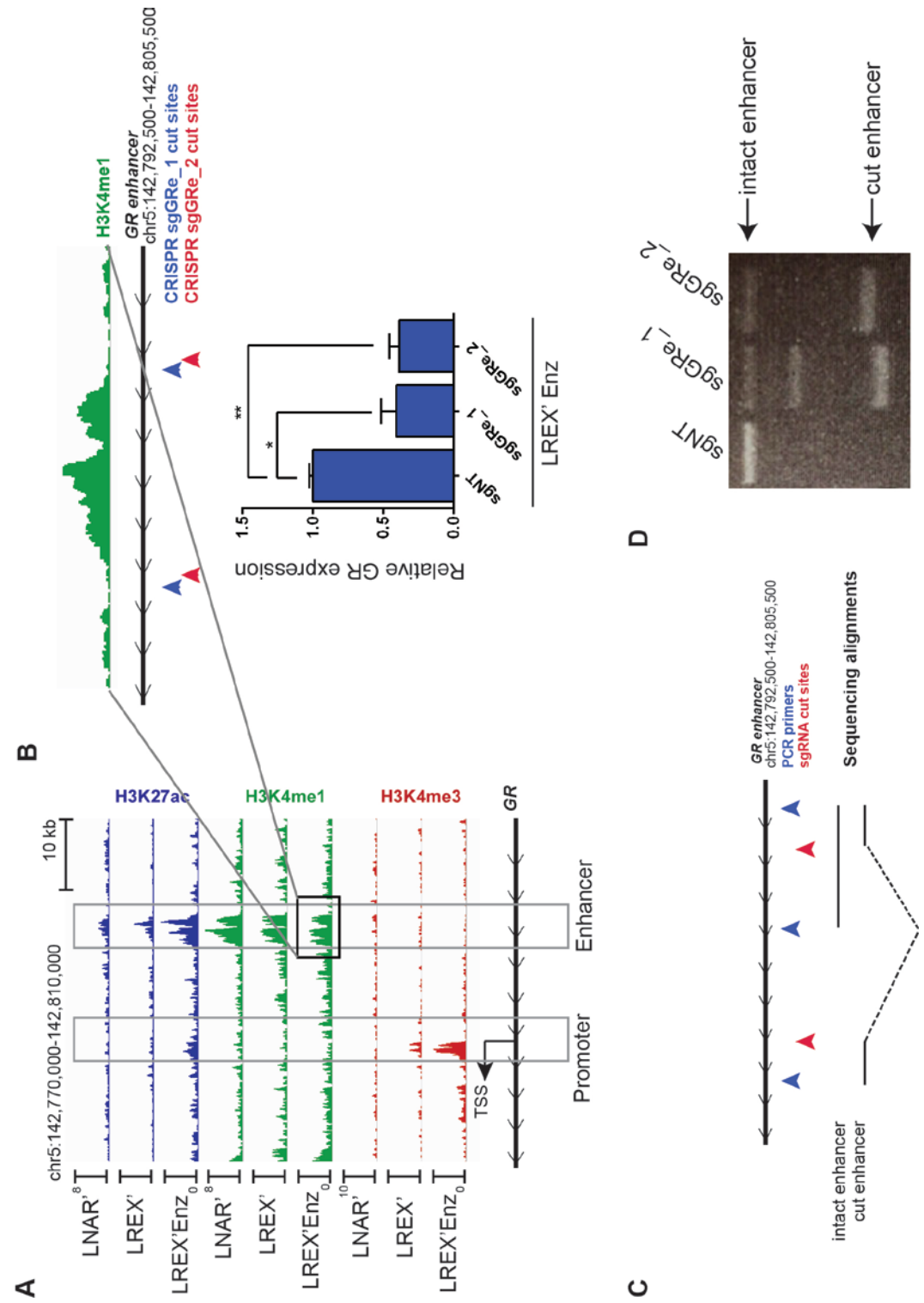

Fig 2.3 - A novel enhancer drives GR expression

(Figure description continued on following page)

Fig 2.3 - (continued)

A) ChIP-seq tracks for H3K27ac (blue), H3K4me1 (green), H3K4me3 (red) at the GR (NR3C1) locus for LNAR', LREX' and LREX'Enz cell lines define the promoter and enhancer regions. Normalized ChIP-seq read counts at GR enhancer/promoter region (LNAR', LREX', LREX'Enz; ** = Z-score > 2): H3K27ac (20.86, 30.57, 72.46**); H3K4me1 (59.62, 36.69, 42.61); H3K4me3 (7.97, 24.54, 94.91**).

B) Top - Experimental design depicting two CRISPR guide pairs (sgGRe_1 and sgGRe_2) flanking the GR (NR3C1) enhancer to excise the entire enhancer region. Bottom - RT-qPCR for GR expression from sorted cells transfected with two different CRISPR guide pairs (sgGRe_1 and sgGRe_2) and a non-targeting (sgNT) control. LREX'Enz cell lines were transiently transfected using Lipofectamine 2000 as per the standard protocol using the PX458 guide pairs, and sorted for GFP expression 4 days post-transfection. GFP-positive cells were then harvested for genomic DNA and RNA using DNEasy kit and RNEasy kit, respectively. Further details are mentioned in Materials and methods section.

C) Sequencing alignments across the GR (NR3C1) locus for different CRISPR guide pairs excising out the GR enhancer region. sgRNA cut sites in red arrows; PCR primers in blue arrows; sequencing alignments as black bars. Further details are mentioned in Material and methods section.

D) DNA agarose gel of PCR products spanning the GR (NR3C1) locus for two different CRISPR guide pairs (sgGRe_1 and sgGRe_2) and a non-targeting control (sgNT). Top band is the intact enhancer PCR product; bottom band is the cut enhancer PCR product.

ChIP-seq analysis and figures created with the help of Ping Wang and Deyou Zheng.

tolerate sustained silencing of GR expression, we deleted the enhancer using a transient approach. LREX' cells were transiently transfected with the guide pairs and a GFP-linked sgRNA-Cas9 vector, then sorted based on GFP expression. PCR analysis of DNA isolated from the GFP-positive population, followed by sequencing, confirmed successful excision of the entire enhancer (Fig 2.3C,D). LREX' cells lacking the enhancer showed a 60 percent reduction in Enz-induced GR expression as measured by quantitative PCR (qPCR) (Fig 2.3B), validating its importance in regulating GR transcription.

Having confirmed the importance of the enhancer in this model, we searched the Roadmap Epigenomics Project and ENCODE databases, together with ChromHMM software which integrates multiple histone modifications (Ernst and Kellis 2012), for evidence of its existence in other tissue types (Fig 2.4). This analysis confirmed active promoters at the GR locus in most tissues, but minimal evidence of a GR enhancer. Exceptions include kidney and liver tissue (and HEPG2 liver cancer cells), with evidence of a weak or poised enhancer, and pancreas and gastric tissue with spotty chromatin marks in this region. One limitation of the Roadmap Epigenomics Project is the lack of any prostate tissue or prostate cell line data. Therefore, we expanded our ChIP-seq analysis of chromatin marks beyond the LNAR'/LREX' model to include additional prostate samples. Due to challenges in obtaining high quality ChIP-seq data from primary prostate tissue, we used organoid cultures to propagate normal and malignant human prostate specimens (Karthaus et al. 2014; Gao et al. 2014). This allowed us to generate high quality ChIP-seq data from two normal human prostate samples (basal and luminal cells) and from the human CRPC organoid line MSK-PCa2 (Gao et al. 2014). We also included publically available ChIP-seq data from the VCaP prostate cancer cell line

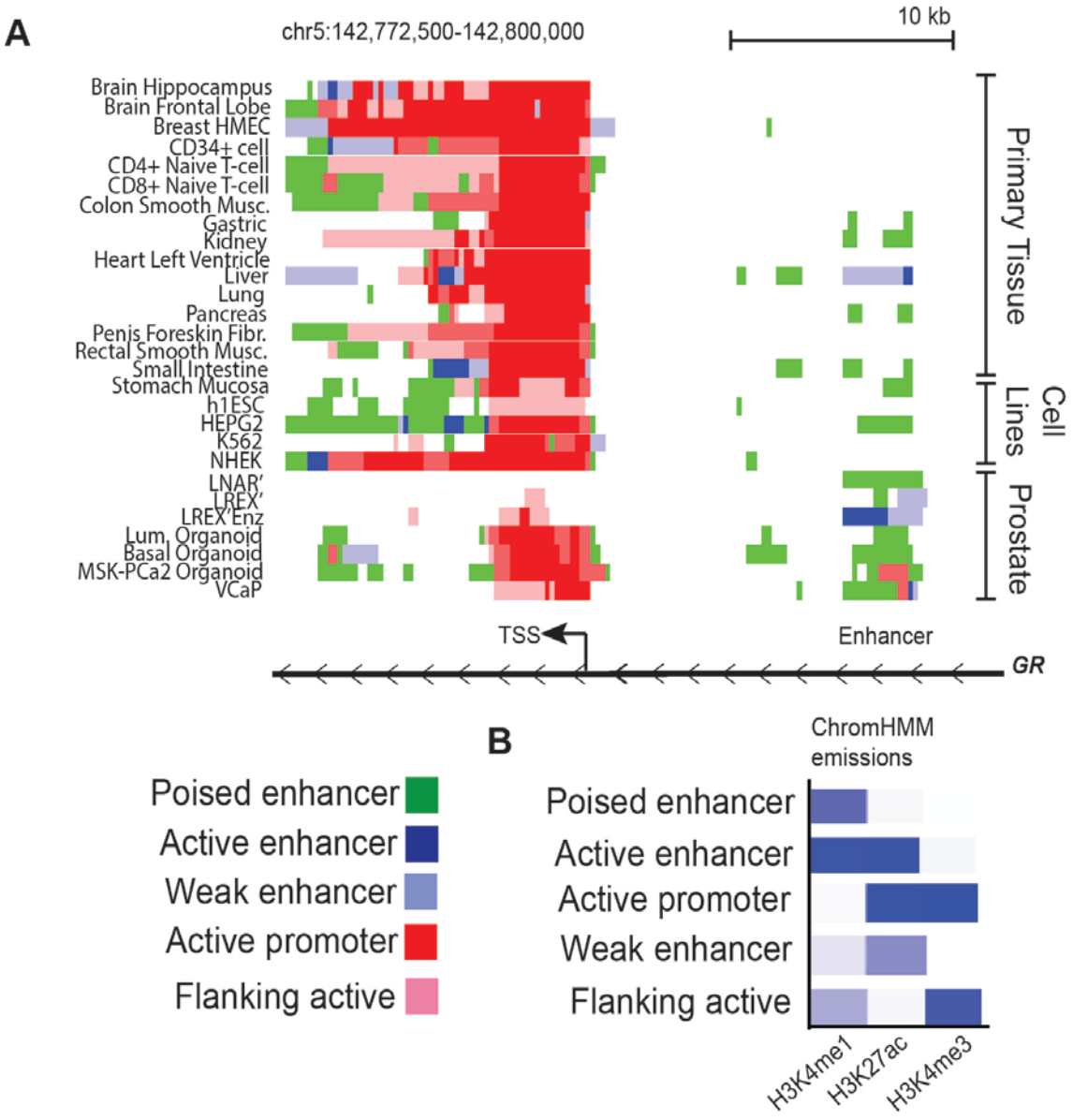

Fig 2.4 - A GR (NR3C1) enhancer that is tissue-specific to the prostate

A) ChromHMM prediction of promoter and enhancer regulatory elements at the GR (NR3C1) locus using ChIP-seq data for H3K27ac, H3K4me1, H3K4me3 across multiple tissue types (from Roadmap Epigenomics Project), cell lines (from ENCODE database), and prostate cell and organoid lines.

(Figure description continued on following page)

Fig 2.4 - (continued)

A) Histone modification ChIP-seq data (H3K27ac, H3K4me1, H3K4me3) of VCaP were downloaded from the Gene Expression Omnibus (GEO). The ChIP-seq data from brain, breast vHMEC, CD34/4/8 cells, colon smooth muscle, gastric, kidney, heart, liver, lung, pancreas, penis foreskin fibroblast primary cells, rectal smooth muscle, small intestine and stomach mucosa were downloaded from the Roadmap Epigenomics project, while the data for h1ESC, HEPG2, K562 and NHEK were from the ENCODE database. Unique reads mapped to a single genomic location were kept. ChromHMM (v1.12) was used to predict chromatin states with default parameters. Further details mentioned in Materials and methods section

B) ChromHMM emissions weighted output of different histone modification marks used in ChromHMM regulatory elements prediction software. Intensity of blue color correlates to importance of the specific histone modification in predicting different regulatory elements.

ChIP-seq analysis and ChromHMM figures created with the help of Ping Wang and Deyou Zheng.

(Yu et al. 2010). In contrast to the non-prostate tissues, this analysis revealed a clearly defined enhancer (H3K4me1) in every prostate tissue examined, including in Enz-treated LREX' cells which also have the active H3K27ac enhancer mark. Taken together, these data reveal an enhancer at the GR locus in normal and malignant prostate cancer cells, not present in most other tissues, that plays a critical role in regulating GR mRNA expression in the Enz-resistant state (Shah et al. 2017).

2.2.2 GR expression in prostate cancer is regulated via AR occupancy at the upstream enhancer and polycomb-mediated silencing

Previous studies have reported GR expression in normal prostate tissue but not in primary prostate cancer (Yemelyanov et al. 2007; Mohler et al. 1996), which we confirmed by immunohistochemistry (IHC) in five tumor-normal pairs. GR is robustly expressed in both basal and luminal cells in normal prostate tissue, but substantially reduced in primary prostate cancer (Fig 2.5A). To examine GR expression across the entire spectrum of prostate cancer from primary to late stage metastatic disease, we conducted a meta-analysis of several large-scale RNA expression datasets (Grasso et al. 2012; Robinson et al. 2015a; Cancer Genome Atlas Research et al. 2013). Consistent with the IHC data, we observed a significant decrease in GR mRNA levels in primary prostate cancer relative to normal tissue, with a further decrease in CRPC samples from patients who have not received Enz or Abi (Fig 2.5B). However, mean GR mRNA levels were significantly increased in CRPC samples obtained after Enz/Abi treatment but not back to the levels seen in primary disease. More detailed analysis of the

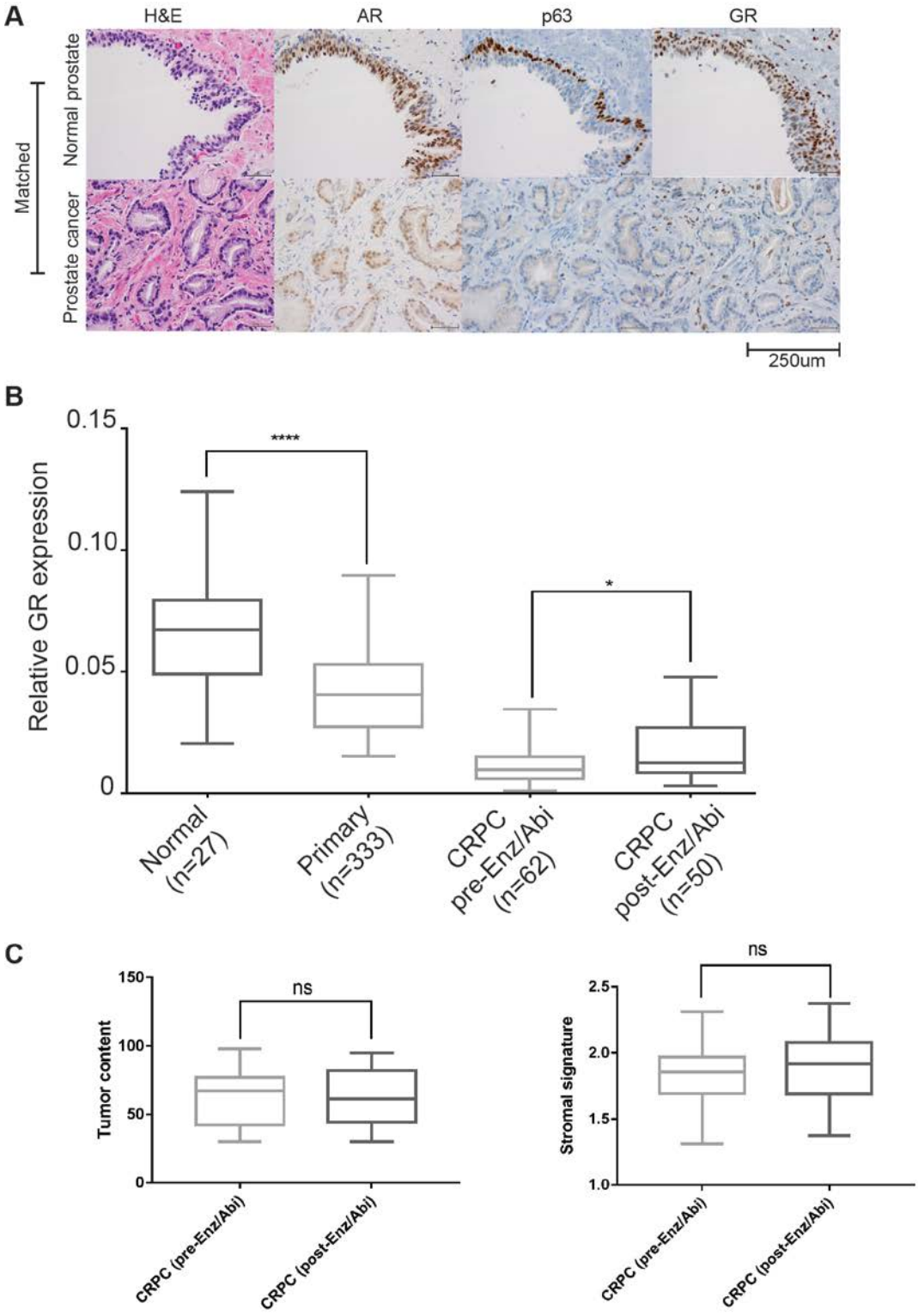

Fig 2.5 - GR expression across multiple prostate cancer disease states

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Fig 2.5 - (continued)

A) Hematoxylin and eosin stain and immunohistochemistry for AR, p63, and GR in a paraffin-embedded patient-matched normal prostate and prostate tumor samples. This figure is representative of five matched normal-tumor pairs, which show the same pattern of staining.

B) Combination of TCGA (normal prostate [n=27] and primary prostate cancer [n=333]) and SU2C (CRPC pre-Enz/Abi [n=62] and CRPC post-Enz/Abi [n=50]) gene expression datasets showing GR expression in prostate cancer disease progression. Boxplot shows median GR expression with 5-95 percentile bars.

Normalized RNA-seq expression values from datasets of The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research et al. 2013) and PCF/SU2C (poly-A RNA-seq samples) (Robinson et al. 2015a) were used. N3RC1 RNA expression was normalized by dividing its expression by the expression of the housekeeping gene UBC. Significantly different values were assessed using Wilcoxon rank sum test. Further details provided in Materials and methods section.

C) Computational tumor purity metrics. Tumor purity content was estimated computationally using the ABSOLUTE method (Carter et al. 2012), based on mutant allele variant fractions and zygosity shifts. Stromal signature score was applied to the normalized RNA-seq expression dataset (Yoshihara et al. 2013). Further details provided in Materials and methods section.

H&E staining was performed with the help of Marina Asher at the MSKCC Histology Core. RNA-seq analysis from genomic datasets was performed with the help of Wassim Abida and Joshua Armenia.

post-Enz/Abi patients revealed that the increase in mean GR level is explained, in part, by a subset of patients with higher GR expression, consistent with our previous finding of increased GR protein expression in a subset of Enz-treated patients (Arora et al. 2013). We also performed computational analyses to compare tumor and stromal cell content and found no differences in the CRPC pre- versus post-Enz/Abi samples (Fig 2.5C).

Next we examined AR mutations, AR splice variants and total AR mRNA levels in Enz/Abi-resistant CRPC tumors and their relationship with GR expression. 7 of 50 post-Enz/Abi samples had detectable AR mutations (all with AR L702H, known to be activated by prednisone and enriched in post-Abi patients) with no correlation with GR levels (Fig 2.6A). The AR-V7 splice variant was negatively correlated with GR expression (Spearman R=-0.39, p=0.03) (Fig 2.6B), suggesting that GR and AR-V7 may define mutually exclusive subsets of Enz/Abi-resistant patients, although larger cohorts are needed for more definitive analysis (Antonarakis et al. 2014; Scher et al. 2016). There was with no significant increase in AR mRNA expression in the pre- versus post-Enz/Abi cases (Fig 2.6C). In summary, this analysis reveals a loss of GR mRNA levels during the transition from normal prostate to prostate cancer, and a subsequent increase in GR expression in the post-Enz/Abi treatment setting.

To explore the mechanism underlying this dynamic regulation of GR expression in prostate cancer progression, we first returned to the LNAR'/LREX' model system where continuous Enz exposure is required to maintain high levels of GR expression. We previously reported that dihydrotestosterone (DHT) treatment suppresses GR expression in LREX cells (Arora et al. 2013) and noted an AR binding peak that, interestingly, maps precisely to this newly defined GR enhancer, adjacent to FOXA1 and HOXB13 motifs

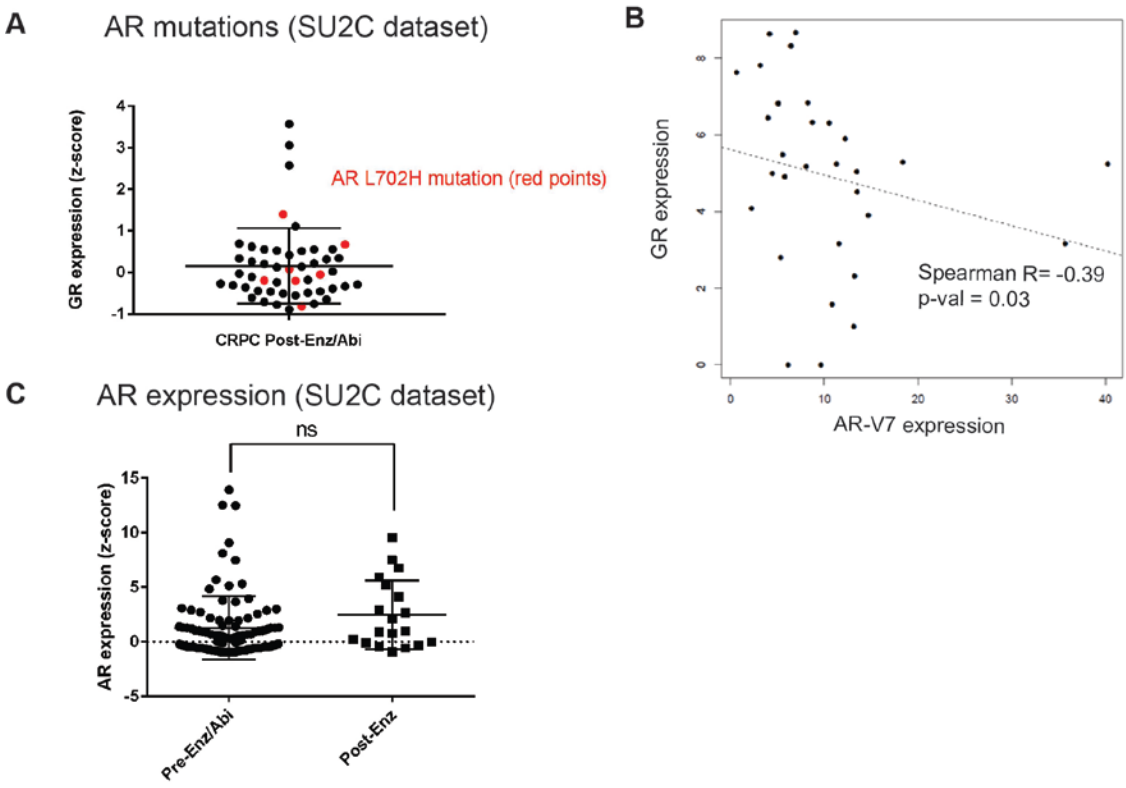

Fig 2.6 - GR expression in relation to other mechanisms of Enz resistance

A) GR expression in individual post-Enz/Abi CRPC tumors. Red dots indicate tumors with AR L702H mutation.

B) Correlation between GR expression and AR-V7 expression in post-Enz/Abi patient samples. AR-V7 expression measured by normalized RNA-seq read counts across splice junction.

C) AR expression in SU2C dataset (26) in CRPC pre-Enz/Abi [n=62] and CRPC post-Enz [n=19] samples.

Sequencing analysis from PCF/SU2C dataset was performed with the help of Wassim Abida and Joshua Armenia.

(Fig 2.7A). We confirmed Enz-reversible AR binding at this site in both LNAR' and LREX' cells by chromatin immunoprecipitation followed by polymerase-chain reaction (ChIP-PCR) (Fig 2.7B). To address the question of how GR expression is silenced in localized prostate cancer, we turned to a set of matched tumor/normal primary clinical samples whose AR ChIP-seq profiles were recently characterized (Pomerantz et al. 2015). Remarkably, AR binding peaks were detected at the GR enhancer in 5 of 5 tumors, but not in the matched normal tissue (Fig 2.7C), precisely correlating with reduced GR mRNA levels seen in primary cancers versus normal tissue. We therefore postulate that AR binding at the GR enhancer site is repressive, as has been reported for an AR binding site within the AR genomic locus (Cai et al. 2014). The differential AR binding at this enhancer in normal versus cancer, despite comparable levels of AR expression, is consistent with extensive reprogramming of the AR cistrome reported in human prostate tumors (Pomerantz et al. 2015).

While repressive AR binding is sufficient to explain reduced GR expression in primary cancer, we postulated that other mechanisms may contribute to the further decline in GR levels observed in CRPC patients. Many promoters and enhancers are regulated through the repressive histone mark H3K27me3 (Rada-Iglesias et al. 2011; Creyghton et al. 2010; Zentner et al. 2011), which is deposited on histones via the EZH2 enzyme, part of the polycomb repressive complex 2 (PRC2). Due to technical challenges in obtaining high quality H3K27me3 ChIP-seq data from primary prostate tissue, we again utilized human prostate organoid cultures to gather data from normal prostate cells and an additional CRPC sample (MSK-PCa2) as well as the isogenic LNAR'/LREX' resistance model. We observed increased H3K27me3 at the GR promoter and enhancer in

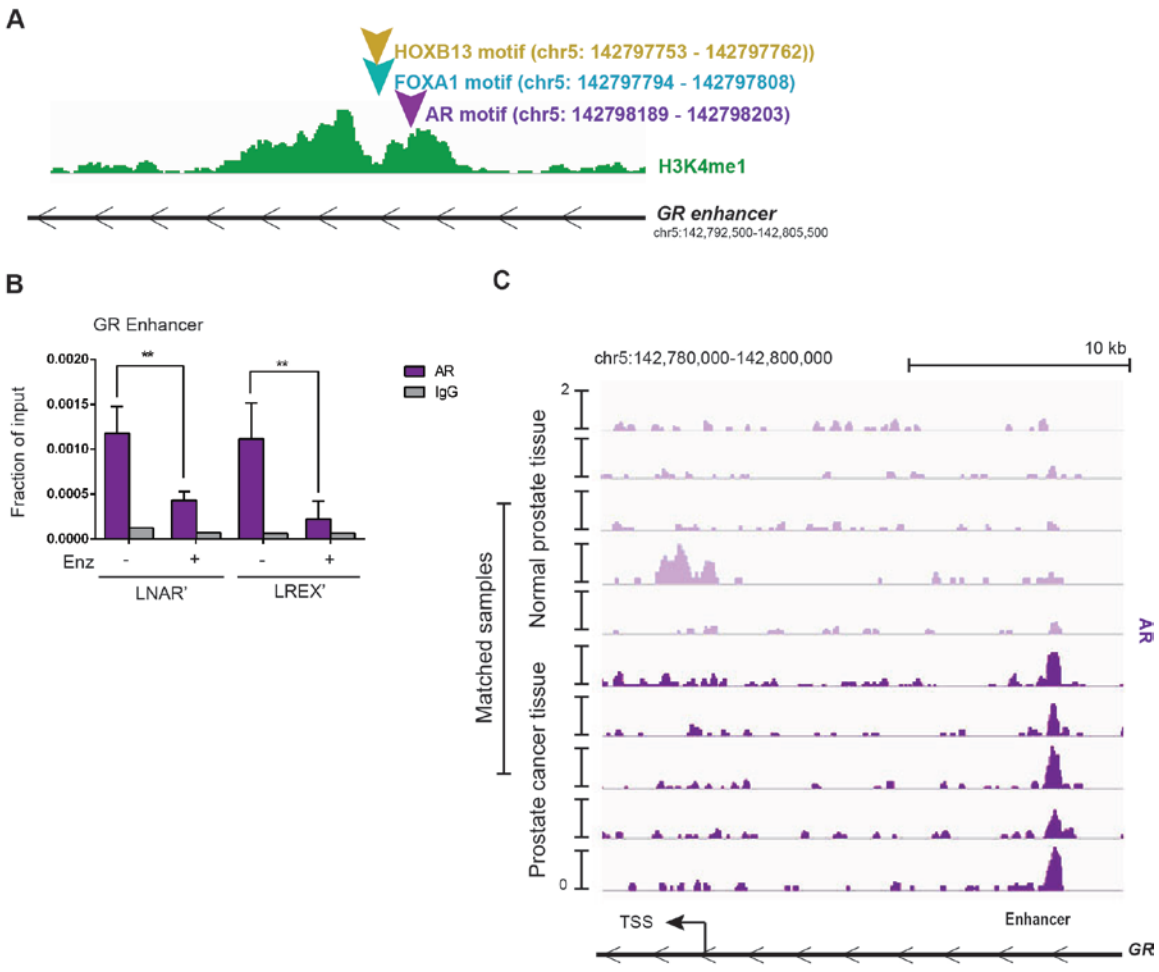

Fig 2.7 - AR binds to the GR enhancer in prostate cancer

A) Transcription factor motif scanning across the GR (NR3C1) locus for HOXB13, FOXA1, and AR.

B) ChIP-qPCR for AR at the GR (NR3C1) enhancer for LNAR' and LREX' cell lines maintained on and off Enz.

C) ChIP-seq tracks for AR at the GR (NR3C1) locus in patient-matched normal prostate tissue and primary prostate cancer tissue samples.

Motif scanning was performed with the help of Ping Wang and Deyou Zheng. AR ChIP-seq analysis from human samples and figures were created with the help of Matthew Freedman and Henry Long.

the cancer samples (MSK-PCa2, LNAR') compared to normal prostate cells, but loss of the H3K27me3 mark in Enz-resistant LREX' cells (Fig 2.8A). The results show that H3K27me3 at the GR locus is correlated with reduced expression. To determine if the H3K27me3 mark is responsible for GR repression, we treated LNAR' cells with the EZH2 inhibitor GSK126 to erase the repressive mark (Fig 2.8B), then measured GR levels in the presence or absence of Enz. Although treatment with GSK126 alone had no effect, GR levels rose more than 30-fold in combination with Enz, to levels comparable to those seen in LREX' cells treated with Enz alone (Fig 2.8C). We observed similar cooperativity between GSK126 and Enz in MSK-PCa2 tumor organoids but not in normal prostate organoids that lack H3K27me3 or AR binding at the GR locus (Fig. 2.8D). Together, these data suggest that GR expression in prostate tissue is silenced during the transition to cancer by a combination of AR binding and PRC2-mediated repression at the GR locus. We postulate that the PRC2 mark can be erased in late stage CRPC (post-Enz/Abi), which enables cells to express GR when exposed to Enz, as Enz treatment displaces AR repression from the GR enhancer (Shah et al. 2017).

2.3 Discussion

It may seem contradictory that GR expression can confer resistance to ADT given the clinical evidence that exogenous glucocorticoid administration can often lead to clinical benefit for CRPC patients. However, this observation is explained by the fact that glucocorticoids inhibit adrenocorticotropic hormone (ACTH) production in the pituitary gland, which subsequently leads to reduced androgen levels (Tannock et al. 1989). This acute reduction of androgen levels can often present as a decrease in the levels of serum

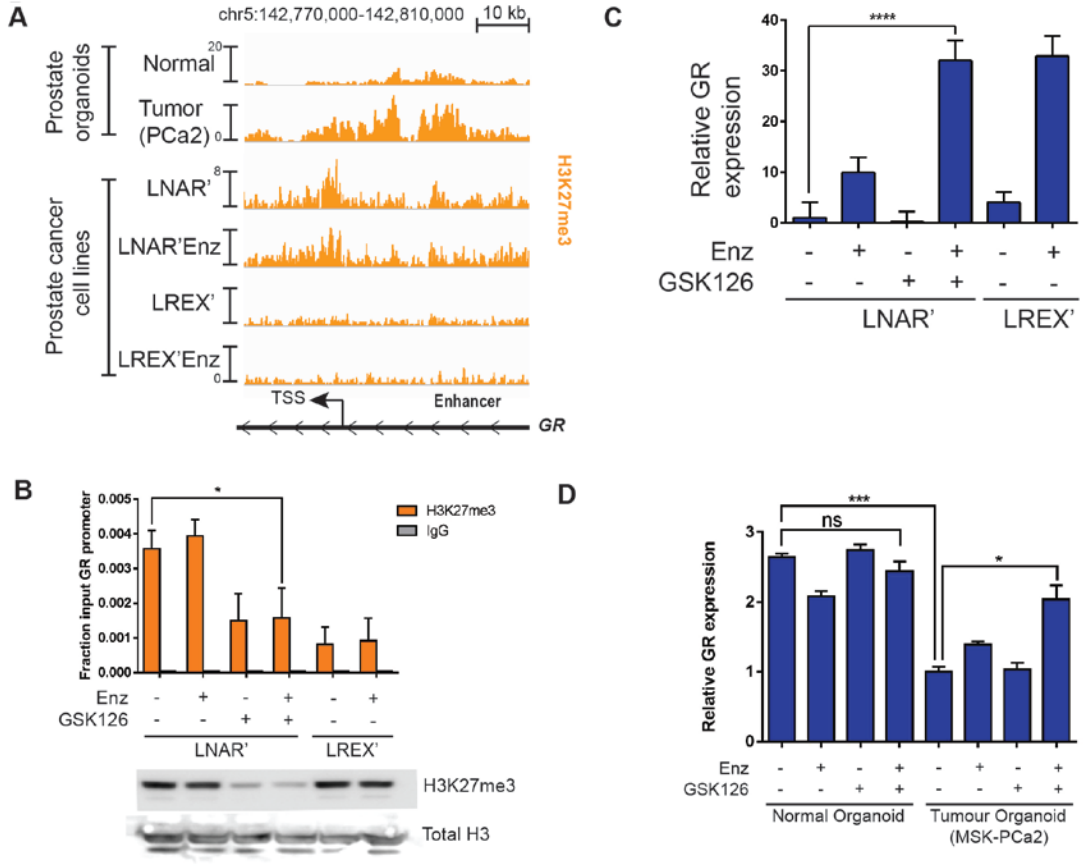

Fig 2.8 - EZH2-mediated repression of GR in prostate cancer

A) ChIP-seq tracks for H3K27me3 at the GR (NR3C1) locus in normal luminal prostate organoids and advanced prostate cancer (MSK-PCa2) organoids (top), and LNAR' and LREX' cell lines maintained on and off Enz (1uM) (bottom). Normalized ChIP-seq read counts at GR enhancer/promoter region (LNAR', LNAR'Enz, LREX', LREX'Enz; ** = Z-score > 2): H3K27me3 (65.44**, 56.99**, 17.97, 8.53). Organoid lines were derived from human normal luminal prostate tissue (CD26+ cells) or human advanced prostate cancer tissue (MSK-PCa2 organoid line), and maintained in 3D culture conditions, as previously described.

B) Top - ChIP-PCR for H3K27me3 at the GR (NR3C1) locus on LNAR' and LREX' cells with different combinations of Enz (1uM) and GSK126 (3uM) over 2 weeks of treatment. Bottom - Western blot for H3K27me3 on LNAR' and LREX' cells with different combinations of Enz (1uM) and GSK126 (3uM) over 2 weeks of treatment.

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Fig 2.8 - (continued)

C) RT-qPCR for GR expression in LNAR' cells treated for 2 weeks with different combinations of Enz (1uM) and GSK126 (3uM), and LREX' cells treated with and without Enz (1uM).

D) RT-qPCR for GR expression in normal luminal prostate organoid and advanced prostate cancer organoid (PCa2) lines treated with different combinations of Enz (1uM) and GSK126 (3uM) over 5 days of treatment.

ChIP-seq analysis and figures created with the help of Ping Wang, Deyou Zheng, Bradley Bernstein, and Yotam Drier.

PSA, as observed in patients taking prednisone (Ryan et al. 2013). However, in patients who have high levels of GR expression in the CRPC setting or those who have relapsed on Enz/Abi, exogenous glucocorticoid treatment may have deleterious effects by driving the growth of these tumors.

The newly defined enhancer that we discovered to regulate GR expression in this model was shown to be tissue-specific to the prostate, and in a poised state. Enhancer elements play an important role in regulating gene expression in different developmental states, and help maintain cell-type identity. Epigenetically, enhancers are defined by the H3K4me1 histone mark, and often exhibit tissue and cell-type specific patterns (Heintzman et al. 2007; Visel et al. 2009). Enhancers can exist in two main states: active or poised. Active enhancers are differentiated from poised enhancers by the presence of a H3K27ac mark in conjunction with the H3K4me1 enhancer mark, while poised enhancers lack this former histone mark (Creyghton et al. 2010; Rada-Iglesias et al. 2011). Poised enhancers can be further demarcated by the polycomb-mediated H3K27me3 repressive mark, emphasizing the importance of tightly regulating and silencing certain genes (Bernstein et al. 2006). It is postulated that poised enhancers contain permissive chromatin dynamics that allow for rapid activation or repression of key genes during development and other tissue processes, and may be important in tumorigenesis and cancer progression (Bonn et al. 2012; Herz et al. 2014).

The fact that this tissue-specific GR enhancer is in a poised state in both normal prostate and primary prostate cancer samples suggests that it may play an important regulatory role. It has been shown that modulation of GR expression and activity can significantly affect normal prostate development and proliferation (Budunova et al. 2004;

Simanainen et al. 2011). However, GR prostate epithelial knockout mice still exhibited corticosterone-induced prostate hyperplasia, suggesting that the stromal GR-expressing tissue or the more basal or neuroendocrine-like prostate cells play a large role in this phenotype (Zhao et al. 2014).

GR regulation via this enhancer is particularly striking in the setting of prostate cancer disease progression, as we see a dramatic decrease of GR expression in primary prostate cancer and CRPC (Shah et al. 2017). This poses the question of why GR is so actively silenced in the transition from normal prostate to prostate cancer? One hypothesis is that in the setting of prostate cancer, which has a drastically distinct AR cistrome and AR-dependence compared to normal prostate epithelium, GR can interfere with optimal AR-signaling. It has previously been shown that exogenous GR expression and activation can exhibit tumor suppressive effects in prostate cancer (Yemelyanov et al. 2007). Furthermore, studies in breast cancer have shown that other nuclear hormone receptors other than the estrogen receptor (ER) can modulate and inhibit ER-binding and transcriptional activity. For example, in ER+ breast cancers, higher GR expression is correlated with a better prognosis, whereas in triple-negative breast cancers, the opposite is true (Pan et al. 2011). This may be explained by the fact that GR can physically bind to and repress ER-bound enhancers, effectively inhibiting ER-driven transcriptional programs (Yang et al. 2017). A similar mechanism is seen between the progesterone receptor (PR) and ER, whereby PR can modulate ER-binding and create a distinct transcriptional signature that is associated with a better clinical prognosis (Mohammed et al. 2015). An analogous mechanism may be prevalent in prostate tumorigenesis: GR is actively silenced to allow AR to be the primary hormone receptor signaling pathway for

prostate cancer growth. However, upon AR inhibition, repression of less optimal transcription factors, such as GR, is alleviated to allow for GR-bypass of the AR blockade. Regardless of the mechanism, it is clear across multiple cancer types that the cross-talk between different nuclear hormone receptors is an important factor in tumorigenesis and disease progression.

3. Targeting GR expression in CRPC via BET bromodomain inhibitors

3.1 Introduction: BET inhibitors in cancer

Enhancer regulation of genes is largely dependent on multiple transcription factors and complexes that allow for promoter-enhancer looping, and promote transcriptional activation and elongation. One such class of proteins is the bromodomain and extraterminal domain (BET) family of epigenetic readers, which includes BRD2, BRD3, BRD4, and BRDT. The BET family of proteins all have two conserved N-terminal bromodomains (BD1 and BD2) which recognize acetylated lysine residues on histone tails (Filippakopoulos et al. 2012). These bromodomain interactions with acetylated lysine motifs, such as H3K27ac, help recruit other regulatory and transcriptional complexes such as P-TEFb and Mediator to help drive gene expression via RNA Pol II elongation (Dey et al. 2009; Jang et al. 2005; Yang et al. 2005).

In cancer in particular, there is growing evidence that inhibition of these BET proteins via a new class of drugs termed BET-inhibitors (BETi) may have important therapeutic potential. Drugs such as JQ1 have a high affinity for the BD1 and BD2 bromodomains of the BET family over other classes of bromodomains (Filippakopoulos et al. 2010). JQ1 specifically binds in the bromodomain pocket in a competitive manner with acetylated histone binding, thus causing the displacement of BET proteins from chromatin upon drug exposure. While BET family proteins, such as BRD4, can be considered general transcriptional regulators, studies have shown that certain areas of the genome are bound by very high levels of BRD4, specifically at important lineage-specific

or active oncogenic enhancers. Genes regulated by these enhancers, often termed 'super enhancers', are particularly sensitive to perturbations by BETi (Loven et al. 2013). As such, BETi via drugs such as JQ1 may have therapeutic potential in targeting key oncogenic drivers in cancer.

For example, in preclinical models of multiple myeloma and certain leukemias, BETi have exhibited compelling anti-tumor activity via downregulation of key oncogenes MYC, IL7R, and BCL2 by suppressing activity at key enhancers bound by high levels of BRD4 (Delmore et al. 2011; Ott et al. 2012; Dawson et al. 2011). These effects are not just limited to hematological malignancies. In lung cancer, sensitivity to BETi is attributed to downregualtion of FOSL1 expression, and in NMYC amplified neuroblastomas via suppression of NMYC transcription (Lockwood et al. 2012; Puissant et al. 2013). Together, this demonstrates the potential for specifically targeting aberrant enhancer activity in treating certain types of cancers. Similarly, Notch-mutant T-cell acute lymphoblastic leukemias (T-ALL) treated with gamma-secretase inhibitors (GSIs) develop an epigenetic, reversible state and subsequent activation of downstream transcriptional programs that bypass the GSI blockade (Knoechel et al. 2014). These leukemias can be targeted via BETi in combination with GSIs, establishing a basis for incorporating epigenetic modulators in combination with targeted therapies to overcome resistance.

Given the high levels of H3K27ac observed at the GR (NR3C1) tissue-specific enhancer described in the previous chapter, we wanted to address if GR was dependent on BET-family transcriptional regulation, if we could modulate GR levels the CRPC

setting, and ultimately, if Enz-resistant cells could be re-sensitized to anti-androgen treatment via the use of BETi.

3.1 Results

3.1.1 BET inhibition impairs GR expression and resensitizes tumors to Enz

Having identified an enhancer responsible for GR upregulation in Enz-resistant CRPC that is marked by H3K27ac, we asked if BET family proteins, which bind acetylated lysine motifs at enhancers and help drive the expression of key tissue-specific genes (Dhalluin et al. 1999; Filippakopoulos et al. 2012; Jang et al. 2005; Shi and Vakoc 2014), regulate GR expression in this setting. Indeed, *in vitro* treatment of LREX' cells with the BET inhibitor JQ1 (Filippakopoulos et al. 2010) resulted in a dose-dependent decrease of GR expression (Fig 3.1A). Consistent with BET bromodomains being epigenetic readers as opposed to writers or erasers, we observed no changes in H3K27ac, H3K27me3 or H3K4me1 at the GR enhancer with JQ1 treatment (Fig 3.1C). BET inhibitors have previously been reported to interfere with AR function, but through a different mechanism, by blocking recruitment of AR to chromatin and impairing expression of downstream target genes rather than directly lowering AR mRNA levels (Asangani et al. 2014; Faivre et al. 2017; Chan et al. 2015); however, we did not observe effects of JQ1 on AR binding at the GR enhancer (Fig 3.1B).

To distinguish between these effects of BET inhibition on GR versus AR (and to determine the impact on Enz-resistance in a GR-driven model), we treated isogenic LNAR’ and LREX’ xenografts in parallel with vehicle, JQ1, Enz or JQ1+Enz, and

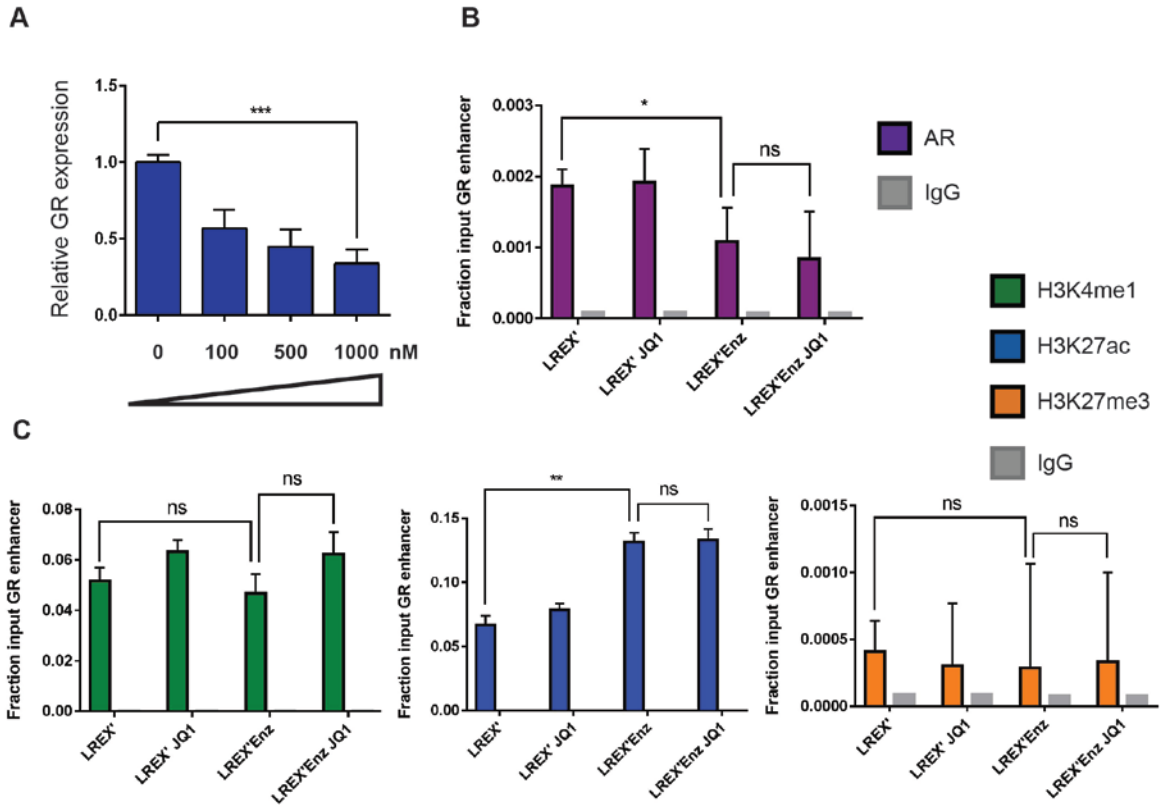

Fig 3.1 - Effects of JQ1 treatment on GR expression and histone modifications

A) RT-qPCR for GR expression in LREX'Enz cells treated in vitro with increasing doses of JQ1 for 24h.

B) Histone ChIP-PCR for AR at GR locus for LREX' cells +/- Enz (1uM) +/- JQ1 (1uM for 48h).

C) Histone ChIP-PCR for H3K4me1, H3K27ac, and H3K27me3 at GR locus for LREX' cells +/- Enz (1uM) +/- JQ1 (1uM for 48h).

performed RNA-seq on the tumors to evaluate effects on gene expression (Fig 3.2). Remarkably, LNAR' tumors showed no response to JQ1 treatment despite retaining their well-documented sensitivity to Enz and dependency on AR-signaling (Fig 3.2A). This is particularly striking because RNA-seq analysis of JQ1-treated LNAR' tumors confirmed that the canonical BET-dependent gene *MYC* was potently downregulated, thereby validating sufficient JQ1 exposure, with no changes in AR target genes such as *NKX3.1* (Fig 3.2B) or an AR target gene signature, as discussed below. In contrast to LNAR' tumors, LREX' tumors were resistant to Enz treatment, as expected. However, LREX' tumors regressed when both JQ1+Enz were delivered in combination (Fig 3B), effectively re-sensitizing resistant tumors to Enz. Of note, some of the most potently downregulated genes in JQ1-treated LREX' tumors were *GR (NR3C1)* itself, the GR target gene *SGK1*, and *MYC*, whereas *NKX3.1* was largely unchanged (Fig 3.2B).

To explore potential reasons for our failure to detect an effect of JQ1 on AR target genes in vivo, we treated LREX' cells with a range of doses (0.01-1uM) *in vitro* and measured impact on several AR versus GR target genes, selected based on previously reported AR-biased versus GR-biased gene signatures (Arora et al. 2013). The AR target genes *NKX3.1* and *TMPRSS2* were upregulated at lower JQ1 concentrations (10nM) but moderately suppressed at higher concentrations (1uM) (Fig 3.3). In contrast, the GR target genes *SGK1* and *FKBP5*, as well as *GR (NR3C1)* itself and *MYC*, were consistently and more profoundly inhibited in a dose-dependent fashion. To further distinguish between the effects of JQ1 on AR versus GR signaling, we performed gene set enrichment analysis (GSEA) on the *in vivo* LREX' tumors. This analysis revealed that Enz primarily inhibits AR-biased genes in LREX' xenografts, whereas JQ1 primarily

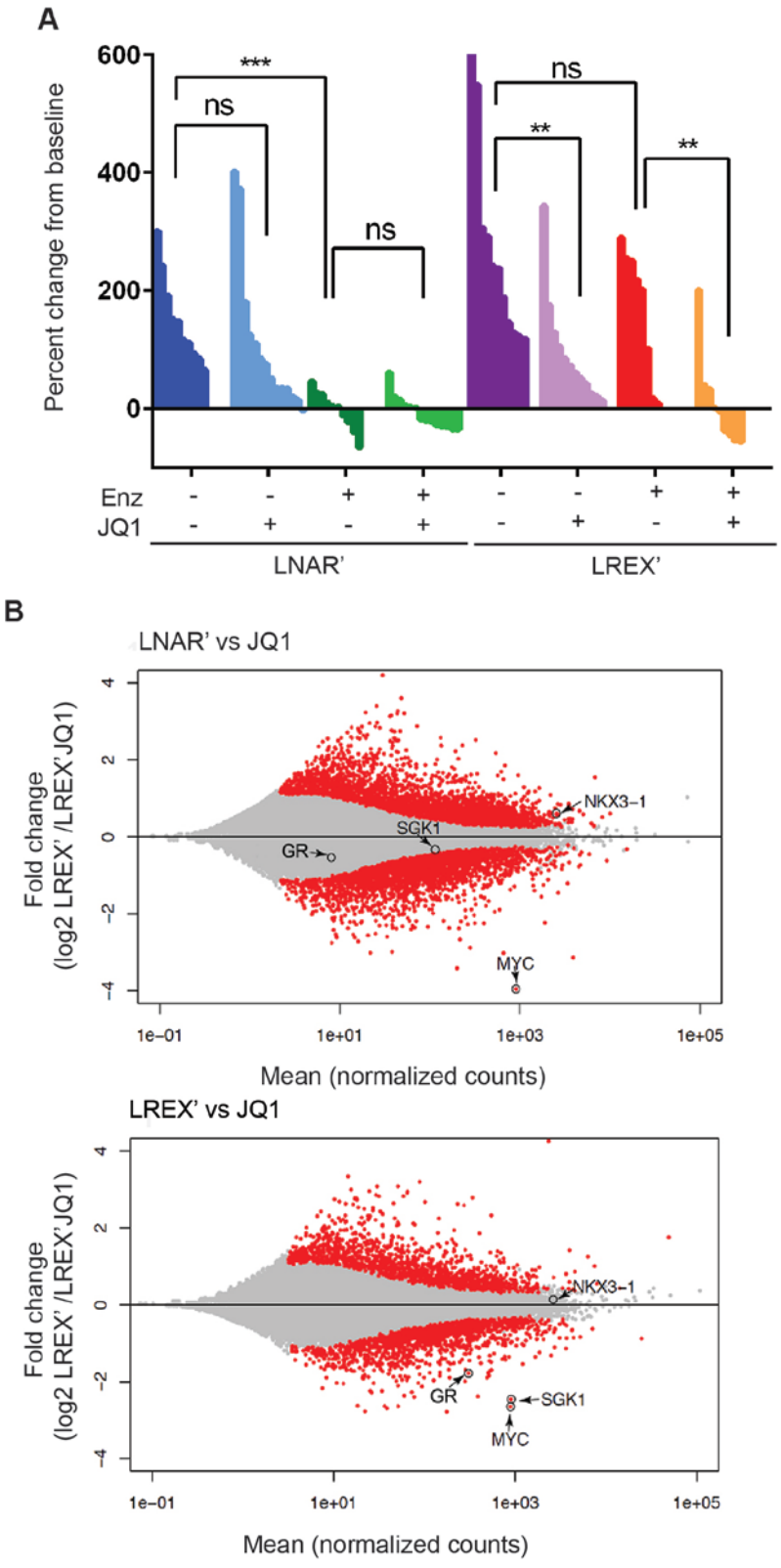

Fig 3.2 - In vivo tumor growth and transcriptome analysis with JQ1 treatment

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Fig 3.2 - (continued)

A) Waterfall plot showing in vivo LNAR' and LREX' xenograft tumor growth, treated with different combinations of Enz (10mg/kg daily) and JQ1(50mg/kg daily) for 4 weeks after tumors are established.

B) MA plot showing differential gene expression analysis from RNA-seq data of in vivo LNAR’ (top) and LREX' tumors (bottom) treated with JQ1 (50mg/kg) after 4 weeks of treatment. Red points are differentially expressed genes (DEGs) that have an adjusted p-val < 0.05. MYC, GR, NKX3.1 (AR target) and SGK1 (GR target) genes are shown on plot.

In vivo tumor xenograft experiments were performed with the help of John Wongvipat and the MSKCC Mouse Core Facility. RNA-seq analysis and figures on resultant tumors performed with the help of Ping Wang and Deyou Zheng.

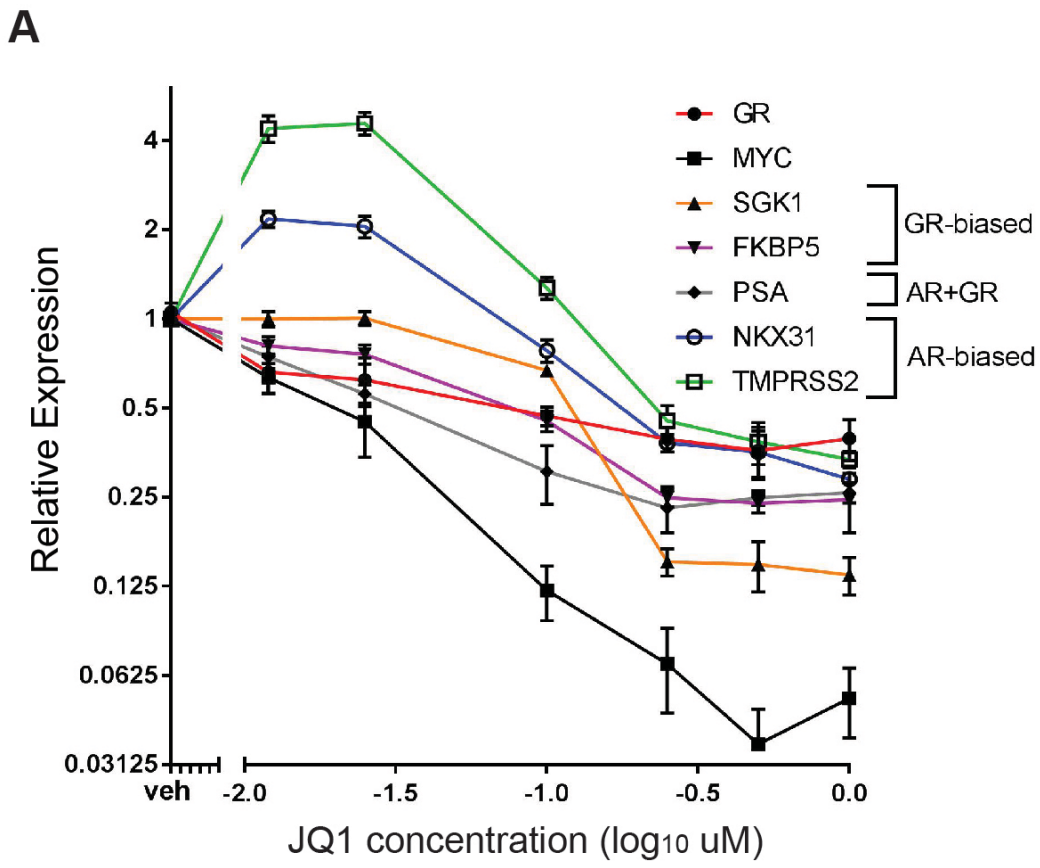

Fig 3.3 - Dose curve of JQ1 on GR and AR targets

A) JQ1 treatment dose-response curve in LREX'Enz cells for 48h, measuring gene expression changes normalized to veh treatment for GR (NR3C1), MYC, SGK1, FKBP5, PSA (KLK3), NKX3.1, TMPRSS2. Error bars represent standard error.

inhibits GR-biased genes (Fig 3.4). It is not until tumors are treated with a combination of JQ1+Enz together do we see inhibition of both AR and GR signaling pathways (Fig 3.4), as well as LREX' tumor regression (Fig 3.2A). The selective effect of JQ1 on GR but not AR in this model is likely explained by the dose-dependent sensitivity of their downstream targets (revealed *in vitro*) coupled with well documented challenges in achieving sustained JQ1 exposure *in vivo* (Matzuk et al. 2012).

To explore the mechanism underlying the relative selectivity of BET inhibition on GR versus AR target genes, we utilized a technique called Chem-seq to map JQ1 interactions with chromatin across the genome using a biotinylated version of the small molecule (bio-JQ1) (Anders et al. 2014). This method has the additional advantage of surveying all JQ1 target proteins at once rather than limiting our analysis to a single factor BET-bromodomain containing family member (Filippakopoulos et al. 2010). As expected, we observed strong bio-JQ1 binding at the MYC gene locus in LNAR' and LREX' cells in the presence or absence of Enz. The specificity of the bio-JQ1 signal was confirmed by BRD4 ChIP-seq which showed binding of the well-known JQ1 target BRD4 at precisely the same locus (Fig 3.5A) as well as by the highly significant correlation of bio-JQ1 with H3K27ac (Fig 3.5B). We also observed significant bio-JQ1 binding at the GR enhancer, but only in LREX' cells treated with Enz, when the H3K27ac mark is present (Fig 3.5A). Interestingly, we did not detect BRD4 binding at the enhancer, suggesting that a different JQ1 target protein is likely responsible for driving GR expression. To address the question of how JQ1 might have relatively selective effects on GR, we conducted an unbiased genome-wide analysis of bio-JQ1 binding in LNAR' and LREX' cells. Remarkably, the GR enhancer is amongst the most differential

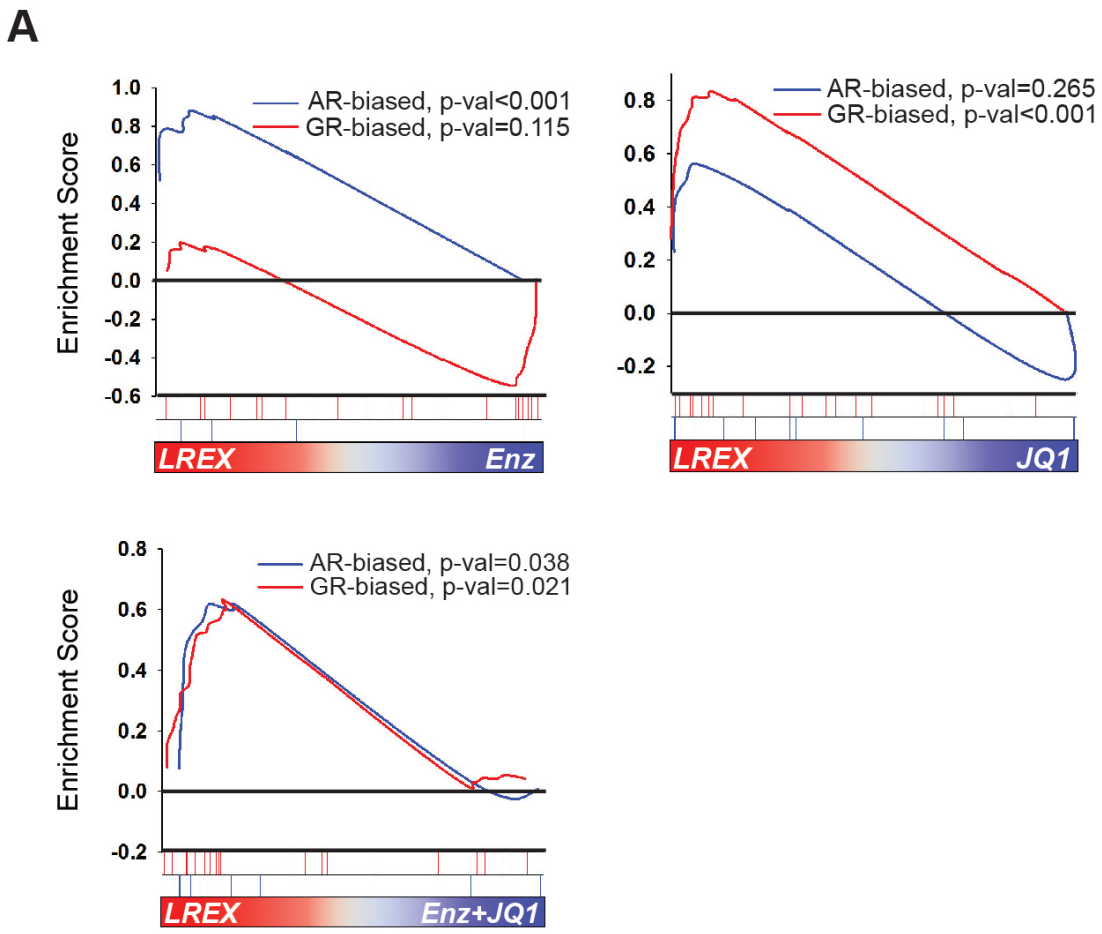

Fig 3.4 - GSEA on JQ1-treated tumors

A) Gene set enrichment analysis (GSEA) using AR-biased and GR-biased gene sets on LREX' tumors treated with Enz (10mg/kg), JQ1(50mg/kg), or a combination of Enz+JQ1 after 4 weeks of treatment. AR-biased and GR-biased gene sets were defined as previously described (Arora et al. 2013), by treating LREX' cells with either 1nM DHT or 100nM Dex, and comparing transcriptional profiles of most up-regulated mutually exclusive genes under each treatment condition.

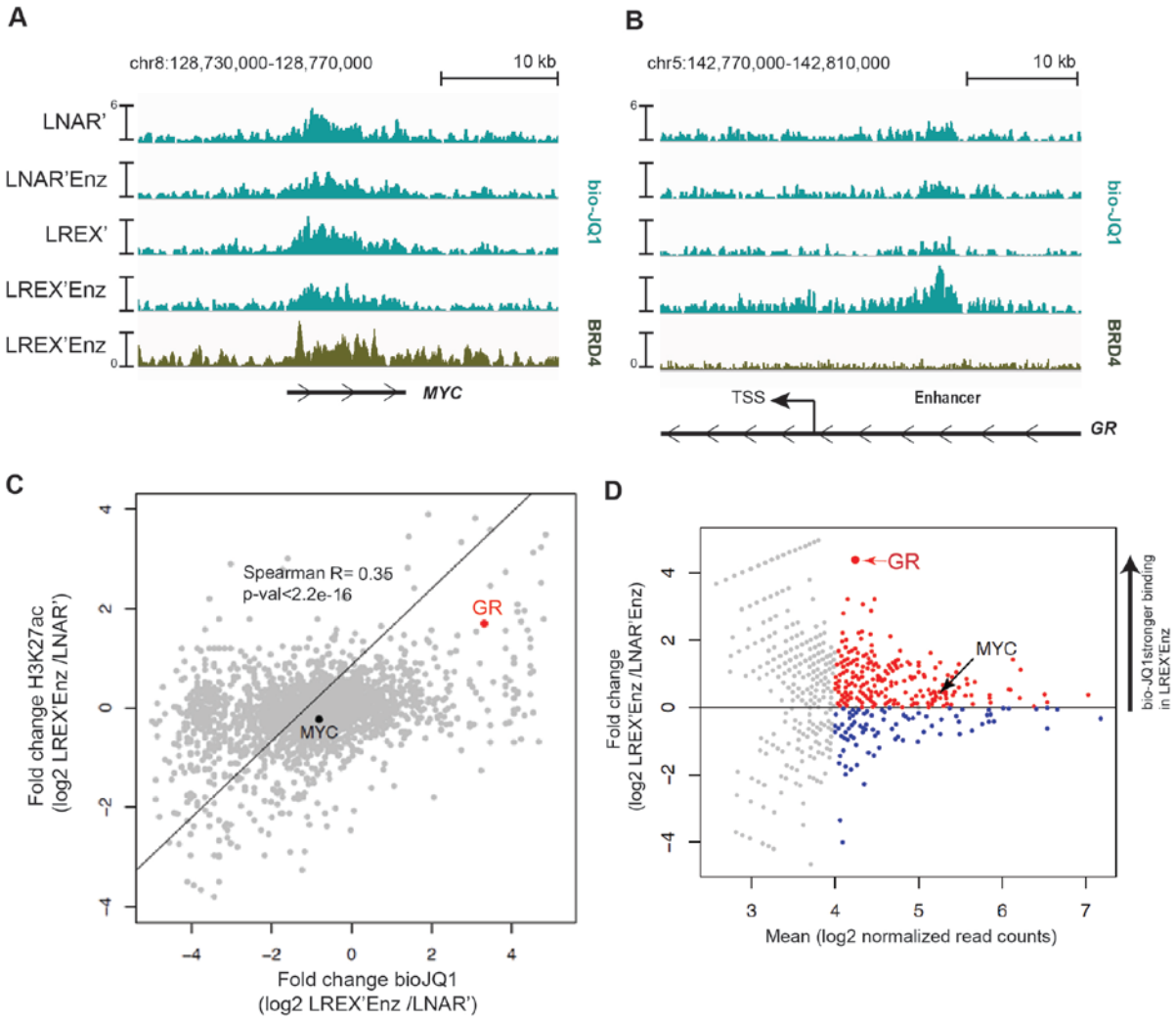

Fig 3.5 - Analysis of JQ1 drug binding

A/B) Chem-Seq tracks for bioJQ1 (light tracks) and ChIP-seq tracks for BRD4 (dark track) in LNAR' and LREX' cell lines treated with and without Enz (1uM), showing the MYC locus (A) and GR gene (NR3C1) locus (B). Normalized Chem-seq/ChIP-seq read counts at MYC promoter region: (LNAR', LNAR'Enz, LREX', LREX'Enz; ** = Z-score > 2): bio-JQ1 (36.53, 48.50, 37.10, 38.49); BRD4 (31.56**). GR enhancer: bio-JQ1 (45.98, 45.46, 33.85, 91.68**); BRD4 (16.49).

B) Correlation of fold change of H3K27ac peaks (between LREX'Enz/LNAR) to fold change of bio-JQ1 peaks. GR and MYC pointed out on plot.

(Figure description continued on following page)

Fig 3.5 - (continued)

C) Differential peak analysis of bio-JQ1 Chem-Seq between LREX'Enz and LNAR'Enz. Colored points have mean log2 normalized reads >4; blue points are bio-JQ1 peaks higher in LNAR'Enz cells, red points are bio-JQ1 peaks higher in LREX'Enz cells. Differential GR and MYC peaks are shown on plot.

Chem-Seq analysis and figures were created with the help of Ping Wang and Deyou Zheng.

binding sites, showing strong preferential binding in LREX’ cells, whereas binding at the MYC locus is unchanged (Fig 3.5D). This enriched binding at the GR locus, together with the primary transcriptional effects on GR and not AR targets, suggests that the antitumor activity of JQ1+Enz in the LREX’ xenograft model is most likely through GR inhibition. To test this hypothesis, we introduced a doxycycline (Dox)-inducible allele of GR under the control of a BET-independent promoter into LREX’ cells and confirmed robust Dox-inducible GR (and downstream SGK1) expression in tumors despite JQ1 treatment (Fig 3.6B,C). Remarkably, GR overexpression in the setting of combined JQ1+Enz rescued tumor growth (Fig 3.6A), thereby demonstrating that the anti-tumor activity of BET inhibition in this model is mediated by GR suppression.

3.3 Discussion

The BETi selectivity for GR inhibition (over AR inhibition) is surprising in light of considerable evidence that BET inhibitors can impair AR function (Asangani et al. 2014; Faivre et al. 2017; Chan et al. 2015). Specifically, it was shown that BETi activity in prostate cancer cells was attributed to direct interference with AR binding to gene loci by perturbing an AR-BRD4 interaction (Asangani et al. 2014). We postulate that the failure to see AR pathway inhibition in our models is due to a dose-dependent, selective inhibition of GR versus AR target genes. It is clear that at lower drug concentrations, we are more effectively able to downregulate GR and subsequent downstream targets over AR targets. The LNAR'/LREX' model has an AR over-expression construct which mimics AR upregulation in the setting of CRPC. Thus, higher AR protein levels, which

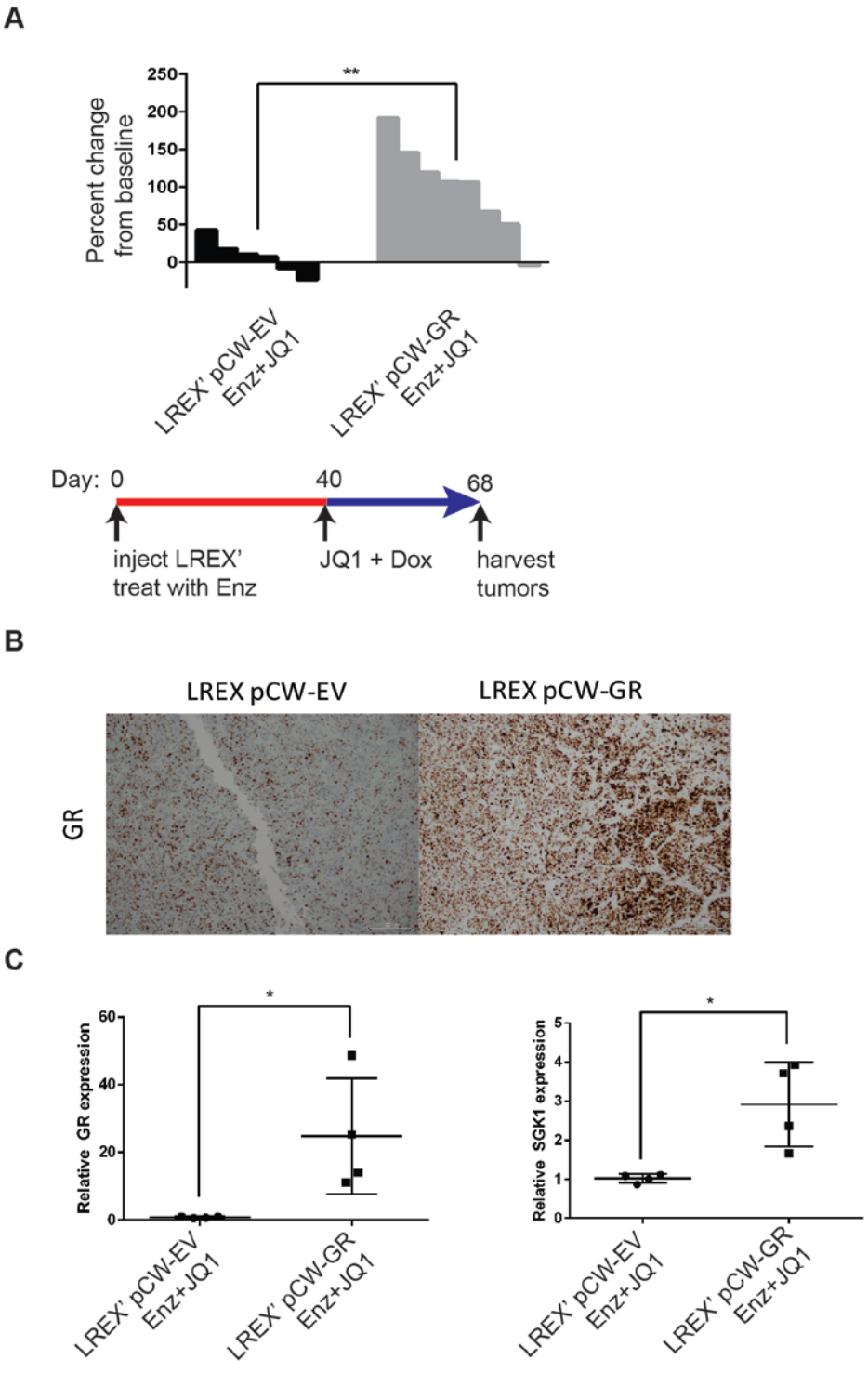

Fig 3.6 - GR rescue of JQ1 sensitivity

(Figure description continued on following page)

Fig 3.6 - (continued)

A) Top - Waterfall plot showing in vivo LREX' xenograft tumor growth with doxycycline-inducible GR over-expression (pCW-GR) or empty vector (pCW-EV) control. Tumors treated with Enz (10mg/kg), JQ1 (50mg/kg), and doxycycline for 4 weeks once tumors are established. Bottom - experimental design for GR overexpression rescue experiment over the course of 68 days.

B) Immunohistochemistry for GR in paraffin-embedded LREX' tumors treated with Enz (10mg/kg), JQ1 (50mg/kg), and doxycycline for 4 weeks, and either an empty vector (pCW-EV) control or doxycycline-inducible GR (pCW-GR) over-expression construct.

C) RT-qPCR for GR and SGK1 in LREX' tumors treated with Enz (10mg/kg) and JQ1 (50mg/kg) over 4 weeks, with either an empty vector (pCW-EV) control or doxycycline-inducible GR (pCW-GR) over-expression construct

In vivo tumor experiments performed with the help of John Wongvipat and the MSKCC Mouse Core Facility. IHC on resultant tumors was performed with the help of Marina Asher at the MSKCC Histology Core.

are often seen in advanced prostate cancers, may be inherently more difficult to perturb with BETi.

Furthermore, differences in the chromatin context of the most BET-dependent genes (e.g. *GR (NR3C1), MYC*) versus AR target genes (e.g. *NKX3.1, TMPRSS2*) may explain the differential binding of bio-JQ1 at these sites as seen by Chem-seq. Prior work, primarily in hematologic malignancies, showing that genes whose enhancers have high levels of H3K27ac (e.g. *MYC*) tend to be exquisitely sensitive to BET inhibition provides additional support to this hypothesis (Shao et al. 2014; Mertz et al. 2011; Ott et al. 2012; Delmore et al. 2011). Regardless of the mechanism, an important implication of this differential sensitivity is that the dose of BET inhibitor required for therapeutic impact is likely to vary depending on the clinical context. In patients with acquired Enz resistance driven by GR, our data predicts that clinical benefit may be seen at lower doses than those required for AR inhibition.

The majority of previous studies on BETi activity in cancer have focused primarily on BRD4. However, while we see clear JQ1 drug binding at the GR enhancer, we do not see any BRD4 binding in the resistant cells. This underscores the importance of looking at all targets of BETi when determining the molecular mechanism of drug action. Furthermore, it may be that BETi drugs, such as JQ1, may not be as specific to BET-family bromodomains as previously thought (Filippakopoulos et al. 2010). Recent efforts have attempted to tease out the role of single BET-family proteins, and address any functional redundancies that may arise between the four proteins: BRD2, BRD3, BRD4, and BRDT (Baud et al. 2014). It has also been shown that BRD2 can play a critical role in numerous cancer types (Coude et al. 2015; Liu et al. 2014). Other

bromodomain containing proteins can also be perturbed by these so-called 'specific' inhibitors, such as ATAD2 in prostate cancer (Urbanucci et al. 2017). It will be interesting to determine which bromodomain-containing proteins are primarily responsible for the BETi sensitivity seen in Enz-resistant prostate cancer cells, which will further shed light on JQ1 specify and anti-tumor activity across multiple cancer types.

While we showed clear sensitivity of LREX' tumors to BETi in combination with Enz, resistance to BETi is an important consideration. It has been shown that hyper-phosphorylation of BRD4 in triple-negative breast cancer cells can lead to resistance to BETi therapy, and these cells are still dependent on BRD4 activity (Shu et al. 2016). In prostate cancers, mutations in SPOP, which is involved in the E3 ubiquitin ligase pathway, can lead to intrinsic resistance to BETi by stabilizing BET protein levels (Zhang et al. 2017; Dai et al. 2017). Given this propensity for BETi resistance, and continued dependence on BET-family proteins, efforts to develop BET-protein degraders are currently in progress (Raina et al. 2016; Bai et al. 2017; Zengerle et al. 2015). In theory, these degraders could overcome the aforementioned mechanisms of resistance to BETi, and may allow for better target protein inhibition.

4. Summary and future directions

In summary, the research presented in this thesis provides new mechanistic insight into how GR expression is restored in a subset of CRPC patients with acquired resistance to Enz, and how BET inhibitors can have surprisingly selective effects on gene expression in specific contexts. Both findings have implications for the future clinical management of CRPC and for the broader topic of reversible and epigenetic mechanisms of acquired drug resistance in cancer.

Based on a careful examination of GR levels across the full clinical spectrum of prostate cancer progression and in several model systems, we find that GR expression in normal prostate epithelial cells is silenced through a two-step process during the transition to malignancy (Fig 3.7). First, we have characterized a novel enhancer at the GR locus, demonstrated that this enhancer is required for GR expression, and shown that AR binding at the enhancer is coupled with reduced GR expression. We propose that the acquisition of AR binding at the enhancer, which triggers a decline in GR expression, is likely a consequence of the reprogrammed AR cistrome in prostate cancer versus normal tissue, which is enriched at HOXB13 and FOXA1 sites (Pomerantz et al. 2015). Second, we have demonstrated increased levels of the repressive H3K27me3 mark across the GR promoter and enhancer in CRPC models, but not in normal prostate tissue organoids, again correlating with reduced GR expression. Increased levels of EZH2 expression observed in CRPC (Varambally et al. 2002) may play a role in the progressive accumulation of the repressive H3K27me3 mark. Finally, pharmacological EZH2

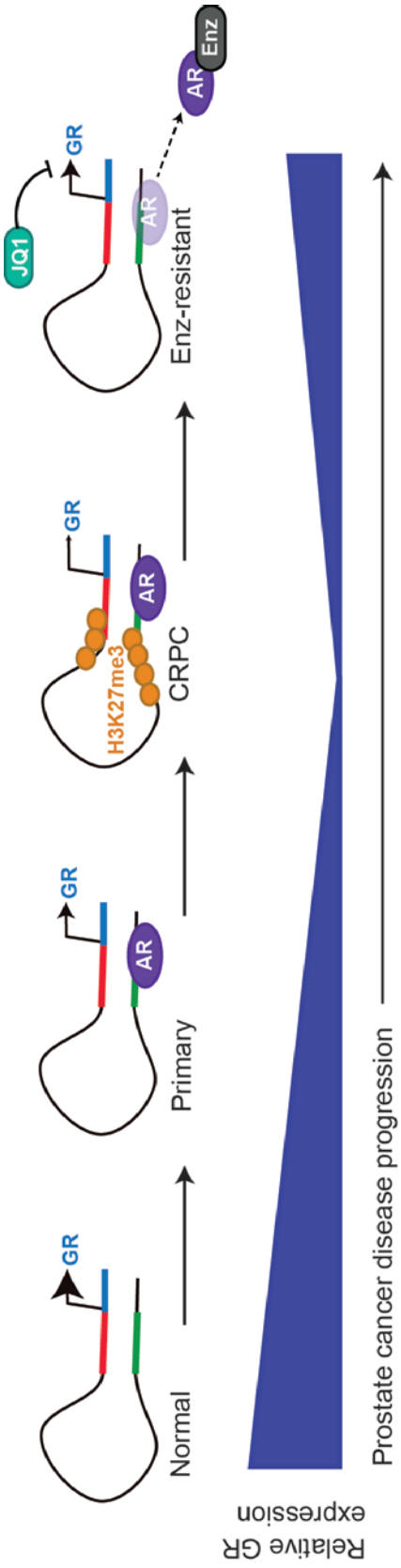

Fig 3.7 - A model for GR regulation in prostate cancer disease progression

inhibition erases the repressive mark and partially restores GR expression, as long as Enz is also present to displace AR repression from the GR enhancer. Collectively, these findings demonstrate that acquired Enz resistance can occur in CRPC patients through reactivation of this normally silenced GR locus. The precise levels of GR re-expression required for Enz resistance will require further study, as well as whether discontinuation of Enz treatment results in a decline in GR levels in patient tumors.

The cross-talk between the nuclear hormone receptors AR and GR is yet to be fully studied, and may shed light as to why GR is so actively silenced in prostate cancer progression and tumorigenesis. Modulation of signaling and binding at similar genomic loci between the two receptors may be potential hypotheses, and similar mechanisms have been observed in breast cancer between the estrogen receptor and numerous other hormone receptors (Yang et al. 2017; Mohammed et al. 2015). Regardless of the mechanism, it is clear that across multiple cancer types that the cross-talk between different hormone receptors is an important factor in disease progression.

The prevalence of GR-driven Enz-resistance in prostate cancer patients remains to be seen. Our research has found that a significant portion of patients do exhibit GR upregulation in the post-Enz setting, however there is a large spectrum of expression, and other mechanisms such as AR mutations/splice variants and lineage-plasticity may also play an important role in resistance to second-generation anti-androgen therapies (Watson et al. 2015). In continuing this research, it will be imperative to obtain patients samples both on and off Enz at the time of progression, as we have clearly shown that AR can bind to and inhibit GR expression at the tissue-specific enhancer.

We also report a remarkably selective effect of BET inhibition in restoring sensitivity to Enz in CRPC by suppression of GR expression through this tissue-specific enhancer, which we demonstrate through a combination of RNA-seq, Chem-seq, and GR rescue experiments. This selectivity for GR inhibition (over AR inhibition) is surprising in light of considerable evidence that BET inhibitors can impair AR function (Asangani et al. 2014; Faivre et al. 2017; Chan et al. 2015). We postulate that the failure to see AR pathway inhibition in our models is due to a dose-dependent, selective inhibition of GR versus AR target genes. Furthermore, while BRD4 binding was not seen at the GR enhancer, it will be important to discover the exact bromodomain containing proteins that are involved in GR transcriptional regulation. Together, these results could provide critical insight into designing future clinical trials to optimize therapeutic impact while avoiding the significant dose-limiting toxicities observed in current single agent BET inhibitor trials (Kharfan-Dabaja 2016).

Numerous BETi clinical trials are currently ongoing or commencing for the treatment of advanced cases of prostate cancer, and it will be worthy to observe these studies and their clinical outcomes. Based on our findings, we believe that BETi as a single agent will not be sufficient to see anti-tumor activity, and will need to be combined with effective ADT such as continuation of Enz. Furthermore, high concentrations of BETi drug required to inhibit AR activity, as reported by others, may not be achievable in vivo, further underscoring the importance of using these drugs in combination with androgen-targeted therapies, particularly in the setting of GR-driven resistance. Regardless of the outcomes, it will be interesting to follow the clinical implications that

the research presented in this report may have on the management and treatment of patients afflicted with prostate cancer.

5. Materials and methods

Cell lines and organoid culture conditions

Cell lines and organoid lines were derived and cultured in conditions as previously described (Karthaus et al. 2014; Gao et al. 2014; Drost et al. 2016; Arora et al. 2013). LNAR' (previously known as CS1 (Arora et al. 2013)) and LREX' cell lines were derived from in vivo xenograft experiments, and adapted back into in vitro culture conditions with or without 1uM Enz. Organoid lines were derived from human normal luminal prostate tissue (CD26+ cells) or human advanced prostate cancer tissue (MSK-PCa2 organoid line), and maintained in 3D culture conditions, as previously described. All cell and organoid lines were authenticated by exome sequencing methods, and were negative for mycoplasma contamination testing.

Intracellular GR staining and flow cytometry

Flow cytometry for GR in LNAR' and LREX' cell lines were performed as previously described (Arora et al. 2013). Cells were fixed and permeabilized using a fixation/permeabilization kit (eBioscience cat no. 00-5523-00), and incubated with either Rabbit (DA1E) mAb IgG XP Isotype control, or glucocorticoid receptor (D6H2L) XP Rabbit mAb (Cell Signaling Technology). Secondary antibody used was Allophycocyanin-AffiniPure F(ab) Fragment Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories). Flow cytometry was performed on LSRII (BD Biosciences), and analysis was conducted using FlowJo software.

Chromatin Immunoprecipitation (ChIP) and sequencing analysis

ChIP experiments were performed as previously described (Arora et al. 2013), using SDS-based buffers. Antibodies were used at a concentration of 5ug per 1mL of IP buffer, which encompassed approximately 8 million cells per IP. Antibodies used were: H3K27ac (Abcam ab4729), H3K4me1 (Abcam ab8895), H3K4me3 (Abcam ab8580), H3K27me3 (Millipore 07-449) AR (Millipore PG-21), BRD4 (Sigma HPA015055).

For ChIP-PCR analysis, the following primers were used: NR3C1 enhancer - F: ACCAGACTGAATGTGCAAGC; R: AGGGTTTTTGATGGCACTGA. For ChIP-Seq, libraries were made using the KAPA Biosystems Hyper Library Prep Kit (cat. # KK8504), using 10ng of DNA as input and 10 PCR cycles for library amplification. The samples were done as single runs per sample on a HiSeq 2500, as rapid run v2 chemistry, single read 50.

Reads were aligned to the human genome (hg19) using Bowtie (v1.1.1) (Langmead et al. 2009) with default parameters and the results were converted to bam files by Samtools (Li et al. 2009). Reads mapped to a single genomic location were kept and redundant ones were filtered out. Tdf files were generated from the bam files by Igvtools with default parameters for visualization in IGV (Robinson et al. 2011).

For organoid ChIP-seq analysis, reads were aligned to the human genome (hg19) using BWA aln (v0.7.4) with default parameters. Reads mapped to more than two genomic loci were filtered out. Tdf files were generated from the bam files by IGV tools with default parameters for visualization in IGV.

ChromHMM GR (NR3C1) locus analysis

Histone modification ChIP-seq data (H3K27ac, H3K4me1, H3K4me3) of VCaP were downloaded from the Gene Expression Omnibus (GEO) (GSM1328982, GSM353631 and GSM353620, respectively). The ChIP-seq data from brain, breast vHMEC, CD34/4/8 cells, colon smooth muscle, gastric, kidney, heart, liver, lung, pancreas, penis foreskin fibroblast primary cells, rectal smooth muscle, small intestine and stomach mucosa were downloaded from the Roadmap Epigenomics project (Roadmap Epigenomics et al. 2015), while the data for h1ESC, HEPG2, K562 and NHEK were from the ENCODE database (Consortium 2012). Unique reads mapped to a single genomic location were kept. ChromHMM (v1.12) was used to predict chromatin states with default parameters (Ernst and Kellis 2012).

CRISPR of GR (NR3C1) enhancer

CRISPR sgRNA guides were cloned into the PX458 plasmid vector (Addgene plasmid #48138) as previously described (Ran et al. 2013). sgRNA primer guide pairs flanking the GR (NR3C1) enhancer were as follows:

sgRNA_1: CACCGTTAATTTCGCCCCCGTCCTG

sgRNA_2: CACCGAATTGTGACTATCAGAGGCT

sgRNA_3: CACCGAGGGGAGGGAATGTACGAAT

sgRNA_4: CACCGGAATTGTGACTATCAGAGGC

LREX'Enz cell lines were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) as per the standard protocol using the PX458 guide pairs, and sorted for

GFP expression 4 days post-transfection. GFP-positive cells were then harvested for genomic DNA and RNA using DNEasy kit and RNEasy kit (Qiagen), respectively.

PCR primers used for amplifying and sequencing the excised enhancer were as follows:

GRe_flank_F- CACACAATCCCATTTTGCAG

GRe_flank_R - TAGCGCTCCCAGGCTTATTA

GR_internal_F - ACCAGACTGAATGTGCAAGC

RT-qPCR analysis

RNA was harvested from cell lines and tumors using RNeasy kit (Qiagen). cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Data was always normalized relative to ACTB. Primers for GR, AR, SGK1, NKX3_1, and ACTB were all purchased through Qiagen.

Analysis of GR expression and AR alterations across multiple datasets

Normalized RNA-seq expression values from datasets of The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research et al. 2013) and PCF/SU2C (poly-A RNA-seq samples) (Robinson et al. 2015a) were used. N3RC1 RNA expression was normalized by dividing its expression by the expression of the housekeeping gene UBC. Significantly different values were assessed using Wilcoxon rank sum test.

AR mutations and AR-V7 expression values were from the PCF/SU2C dataset. AR-V7 expression measured by normalized RNA-seq read counts across splice junction.

Tumor purity content was estimated computationally using the ABSOLUTE method (Carter et al. 2012), based on mutant allele variant fractions and zygosity shifts. Stromal signature score was applied to the normalized RNA-seq expression dataset (Yoshihara et al. 2013).

In vivo xenograft experiments

Tumors xenograft experiments were performed as previously described (Arora et al. 2013). 2 million cells were injected subcutaneously into the flank of castrated CB17 SCID mice in a 50:50 mix of matrigel and regular culture medium. Measurements were obtained weekly using Peira TM900 system (Peira bvba, Belgium). All animal experiments were performed in compliance with the guidelines of the Research Animal Resource Center of Memorial Sloan Kettering Cancer Center. Drug treatments included: enzalutamide (10mg/kg in vehicle 1% carboxymethyl cellulose, 0.1% Tween-80, 5% DMSO) 5 days a week by oral gavage; JQ1 (50mg/kg in vehicle 10% hydroxypropyl beta cyclodextrin) 5 days a week by intraperitonial injection; doxycycline treatment provided through water and food. Tumors were started on treatment once they reached a sufficient size ($200mm^3$), and harvested 4 weeks post-treatment start.

Transcriptome analysis

RNA was harvested from cell lines and tumors using RNeasy kit (Qiagen). RNA sequencing libraries were prepared using the KAPA Hyper Prep Kit (kapabiosystems) in accordance with the manufacturer's instructions. Briefly, 40ng (or less if not available) of total RNA was used for cDNA synthesis and amplification using the Ovation RNA-Seq

System (Nugen). cDNA then was adenylated, ligated to Illumina sequencing adapters, and amplified by PCR (using 10 cycles). Final libraries were evaluated using fluorescent-based assays including PicoGreen (Life Technologies) or Qubit Fluorometer (invitrogen) and Fragment Analyzer (Advanced Analytics) or BioAnalyzer (Agilent 2100), and were sequenced on an Illumina HiSeq2500 sequencer (v4 chemistry) using 2 x 50bp cycles.

Reads were aligned to the human genome (hg19) using STAR (v2.4.2a) (Dobin et al. 2013). The number of RNA-seq fragments mapped to each gene was determined for all genes in the GENCODE database (v19) using the HTSeq (Harrow et al. 2012; Anders et al. 2015). DESeq2 was used to determine differentially expressed genes at false discovery rate < 0.05 (Love et al. 2014).

Gene set enrichment analysis (GSEA)

GSEA analysis was performed from gene sets that were adapted from a method as previously described (Arora et al. 2013) using software publicly available from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). The gene sets used were as follows: AR-biased gene set - NKX3-1, PIK3AP1, TIPARP, PRAGMIN, DKFZP761P0423, ENDOD1, SLC2A3, C1ORF116, TMPRSS2, PAK1IP1, CROT, FZD5, GADD45G, ZNF385B, SLC36A1; GR-biased gene set - SGK1, TUBA3E, SCNN1G, TUBA3C, DDIT4, EMP1, KRT80, TUBA3D, ACTA2, RGS2, C9ORF152, PNLIP, PPAP2A, SLC25A18, S100P, SPSB1, HSD11B2, LOC440040, SPRYD5, TRIM48, KLF9, PGC, LOC340970, ZNF812, PRR15L, PGLYRP2, BCL6, LOC399939, AZGP1, PRKCD, LOC100131392, GADD45B, ZBTB16, EEF2K, CRY2, LIN7B, KIAA0040, FKBP5, STK39, CGNL1, MT1X

Chem-Seq and analysis

Chem-seq was performed as previously described using biotinylated JQ1 compound (Anders et al. 2014). Library preparation and sequencing was performed as described earlier, same as ChIP-seq protocol. Reads were aligned to the human genome (hg19) using Bowtie. Non redundant reads mapped to a single genomic location were kept for peak calling by the MACS2 (v2.1.0) software (Zhang et al. 2008), with parameter –q 0.5. Peaks of differential binding were determined by DiffBind (Ross-Innes et al. 2012).

Tumor xenograft and tissue microarray IHC

Immunohistochemsitry (IHC) was performed on tumor xenografts and tissue microarrays as previously described (Arora et al. 2013). Tumors were fixed in 4% PFA prior to paraffin embedding and then were stained for GR at 1:200 with anti-glucocorticoid receptor (D6H2L) XP Rabbit mAb (Cell Signaling Technology, #12041) using the Ventana BenchMark ULTRA.

Statistics

All RT-qPCR and xenograft volume change comparisons are by two-sided t test. In meta-analysis across multiple large RNA-seq datasets, significantly different values were assessed using Wilcoxon rank sum test. For in vivo tumor transcriptome analysis, DESeq2 was used to determine differentially expressed genes at false discovery rate < 0.05. Four tumors were used per tumor condition. GSEA statistical analysis was carried out with publicly available software from the Broad Institute

(http://www.broadinstitute.org/gsea/index.jsp). In all figures, $*p = < 0.05$, $**p = < 0.01$, $***p = < 0.001$, and $****p = < 0.0001$.

For Z-score ChIP-seq statistics, loci in figures were split into bins, and normalized read counts (per 10 million reads) were calculated for each bin. Z-scores were calculated to find bins with more ChIP-seq reads than the rest in each sample.

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