

Targeting bromodomain and extra-terminal (BET) family proteins in castration resistant prostate cancer (CRPC)

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Statement of Translational Relevance

Advanced prostate cancer invariably progresses to lethal castration resistant prostate cancer (CRPC). Resistance to current androgen receptor (AR) targeting therapies is associated with the development of AR aberrations including the constitutively active AR splice variant 7 (AR-V7). Currently, no clinically available therapies effectively inhibit aberrant AR signaling. BRD4, a bromodomain and extra-terminal (BET) family protein, is a critical AR coregulator. We show that BRD4 expression associates with patient outcome and AR driven transcription in lethal prostate cancer. Moreover, BET inhibitors (BETi) reduce AR splicing and AR-V7 expression by regulating alternative splicing, abrogating AR signaling and inhibiting growth of CRPC patient derived models. Clinical studies with BETi in CRPC should pursue pharmacodynamics studies evaluating abrogation of AR splicing and persistent AR signaling to optimize the development of these drugs for the treatment of CRPC.

Abstract

Purpose: Persistent androgen receptor (AR) signaling drives castration resistant prostate cancer (CRPC) and confers resistance to AR targeting therapies. Novel therapeutic strategies to overcome this are urgently required. We evaluated how bromodomain and extra-terminal (BET) protein inhibitors (BETi) abrogate aberrant AR signaling in CRPC.

Experimental Design: We determined associations between BET expression, AR driven transcription and patient outcome; and the effect and mechanism by which chemical BETi (JQ1 and GSK1210151A; I-BET151) and BET family protein knockdown regulates AR-V7 expression and AR signaling in prostate cancer (PC) models.

Results: Nuclear BRD4 protein expression increases significantly ($p < 0.01$) with castration resistance in same patient treatment naïve (median H-score; interquartile range: 100; 100-170) and CRPC (150; 110-200) biopsies, with higher expression at diagnosis associating with worse outcome (HR 3.25, 95% CI 1.50-7.01; $p < 0.001$). BRD2, BRD3 and BRD4 RNA expression in CRPC biopsies correlates with AR driven transcription (all $p < 0.001$). Chemical BETi, and combined BET family protein knockdown, reduce AR-V7 expression and AR signaling. This was not recapitulated by C-MYC knockdown. In addition, we show that BETi regulates RNA processing thereby reducing alternative splicing and AR-V7 expression. Furthermore, BETi reduce growth of PC cells and patient derived organoids with known AR mutations, AR amplification and AR-V7 expression. Finally, BETi, unlike enzalutamide, decreases persistent AR signaling and growth ($p < 0.001$) of a patient derived xenograft model of CRPC with AR amplification and AR-V7 expression.

Conclusion: BETi merit clinical evaluation as inhibitors of AR splicing and function, with trials demonstrating their blockade in proof of mechanism pharmacodynamic studies.

1. Introduction

Prostate cancer (PC) is the most common cancer and second leading cause of cancer related death in men in the Western world (1). The androgen receptor (AR) remains the main oncogenic driver for PC development and progression (2-5). Despite initial robust responses to androgen deprivation therapy (ADT), nearly all patients with advanced disease progress to fatal castration resistant PC (CRPC). There is increasing evidence of persistent AR signaling as patients progress to CRPC with rising prostate specific antigen (PSA), increasing steroidogenesis, overexpression of AR co-regulators and the development of AR aberrations (6-8). The discovery of second generation anti-androgen therapies, such as abiraterone and enzalutamide, that effectively target AR signaling in patients with hormone sensitive disease and CRPC has improved patient outcome (9-13). However, primary and secondary resistance to both therapies is common and may, in part, be due to the expression of constitutively active AR splice variants of which AR variant 7 (AR-V7) is considered the most significant and best studied (14-27). Consistent with this, recent studies have demonstrated that AR-V7 expression increases as patients progress to CRPC, and associates with resistance to current AR directed therapies (14, 15, 22-26). AR-V7 lacks the AR ligand binding domain (LBD), but contains a cryptic exon 3 (derived from an intron) after AR exon 3 (27). Despite lacking a LBD, AR-V7 continues to signal through its transcriptionally active N-terminal domain facilitating ligand independent AR signaling in CRPC and treatment resistance (17-19, 27). Currently, all licensed therapies that modulate AR signaling do so through the AR LBD and have little or no activity against AR-V7. The development of therapies that overcome aberrant AR signaling in CRPC is an unmet medical need for treating lethal PC.

A promising therapeutic approach, currently undergoing clinical evaluation in CRPC patients, is to target the bromodomain and extra-terminal (BET) family of chromatin readers that include the bromodomain containing protein (BRD) 2, BRD3 and BRD4 (28). BRD4 is a critical coregulator of AR (29, 30). Furthermore, increased BRD4 protein stability in substrate-binding adaptor speckle-type POZ protein (SPOP) mutant prostate cancers is associated with AR signaling (31, 32). BET inhibitors (BETi) and proteolysis targeting chimera (PROTAC) induced BET protein degraders have anti-tumor activity in CRPC models (29, 30, 33-36), attenuating AR signaling and potentially reducing C-MYC expression (29, 30, 33, 34, 36). More recently, BETi have been shown to decrease AR-V7 expression by regulating splicing factors required for its generation (30). Taken together, these preclinical data now need to be validated in prospective clinical trials that evaluate BETi as a therapeutic strategy to overcome persistent AR signaling in CRPC.

Here, we confirm that nuclear BRD4 protein expression increases with castration resistance and associates with patient outcome. Moreover, BRD2, BRD3 and BRD4 RNA expression associates with AR driven transcription in CRPC clinical tumor biopsies. We show that BETi reduce AR-V7 protein expression (as does BET family protein siRNA knockdown) by regulating genes critical for RNA processing and alternative splicing. Finally, we demonstrate that BETi, unlike enzalutamide, regulate AR aberrant (driven by AR mutations, AR amplification and AR-V7 expression) signaling and inhibit the growth of patient derived models of CRPC. These data

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support further investigation of BETi in patients suffering from CRPC with persistent AR oncogenic signaling following next generation AR antagonists.

2. Materials and Methods

Cell lines and drugs

All cell lines used in this study were grown in recommended media at 37 °C in 5 % CO₂ and are detailed in **Supplementary Table 1**. All cell lines were tested for mycoplasma using the VenorGem One Step PCR Kit (Cambio) and STR-profiled using the Cell authentication service by Eurofins Medigenomix. JQ1 was obtained from Stratech Scientific and GSK1210151A (I-BET151) was a kind gift from GlaxoSmithKline (GSK).

Small interfering RNA (siRNA)

Cells were transiently transfected with siRNA as indicated. All siRNA were ON-TARGETplus pools (Dharmacon; GE healthcare), listed in **Supplementary Table 2**. The siRNA was used along with 0.4% RNAiMax transfection reagent (ThermoFisher Scientific) as per manufacturer's instructions and incubated with cells as indicated.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cell line RNA was extracted using the RNeasy Plus Mini kit (Qiagen) as per manufacturer's instructions. Patient derived xenograft (PDX) RNA was obtained by mechanical homogenization, reconstituted with RNeasy RLT buffer, passed through a Qias shredder tube (Qiagen) and further processed with RNeasy Plus Mini kit as above. cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche). qRT-PCR was carried out on ViiA™ 7 System Real-Time PCR System (Life Technologies) using the TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes (ThermoFisher Scientific) used are listed in **Supplementary Table 3**. Fold change in mRNA expression levels was calculated by the comparative Ct method, using the formula $2^{-(-\Delta\Delta Ct)}$.

Western blot

Cells were lysed with RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche). PDX lysate was obtained by mechanical homogenization, reconstituted in RIPA buffer. Protein extracts (20 µg) were separated on 4-12% NuPAGE® Bis-Tris gel (Invitrogen) by electrophoresis and subsequently transferred onto Immobilon-P™ PVDF membranes of 0.45 µm pore size (Millipore). Details of primary antibodies used are provided in **Supplementary Table 4**. Chemiluminescence was detected on the Chemidoc Touch imaging system (Bio-Rad).

Growth assays

Growth of cells was determined using a modified sulforhodamine B assay (SRB) described in **Supplementary Methods** (37).

RNA-sequencing, pathway analysis and alternative splicing events

Cell RNA was extracted from cells using the RNeasy Plus Mini kit (Qiagen) as per manufacturer's instructions. RNA quality was analyzed using Agilent Bioanalyzer RNA Nano kit. 500 ng of total RNA from each sample was used for Illumina's TruSeq Stranded mRNA library prep kit. Library quality was confirmed using the Agilent Bioanalyzer High Sensitivity DNA Assay. The libraries were quantified and normalized by qPCR using KAPA Library Quantification Kit (Roche). Library clustering was performed on a cBot with Illumina HiSeq PE Cluster kit v3. The libraries were sequenced as paired-end 101 base pair reads on an Illumina HiSeq 2500 with an Illumina HiSeq SBS kit v3. Base calling and quality scoring were performed using Real-Time Analysis (version 1.18.64) and FASTQ file generation and de-multiplexing using CASAVA. The paired end raw reads in FASTQ format were aligned to the reference human genome (hg19) using RNA sequencing spliced read mapper TopHat (v2.0.7), with default settings (38). The library and mapping quality were estimated using Picard tools (<http://broadinstitute.github.io/picard>). The alternative splicing events (skipped exons, alternative 5' splice sites, alternative 3' splice sites, mutually exclusive exons and retained introns), based on Ensembl v61 annotation, were accessed using MATS v3.0.8 (39).

Gene expression and androgen receptor (AR) activity score

Data from 122 CRPC transcriptomes generated by the International Stand Up To Cancer/Prostate Cancer Foundation (SU2C/PCF) Prostate Cancer Dream Team were downloaded and re-analyzed (3). Paired-end transcriptome sequencing reads were aligned to the human reference genome (GRCh37/hg19) using Tophat2 (v2.0.7). Gene expression, Fragments Per Kilobase of transcript per Million mapped reads (FPKM), was calculated using Cufflinks (40). AR activity score is an accumulation measurement of AR pathway activity based on 43 genes (**Supplementary Table 5**) regulated by AR in prostate cancer cell line and metastatic prostate cancer (5, 41).

Patient derived xenograft (PDX) development CP50

A metastatic lymph node biopsy from a patient with CRPC who had received all standard of care therapies for prostate cancer was divided and implanted subcutaneously into 2 non-obese diabetic (NOD) scid gamma (NSG) (JAX Mice) male mice (termed CP50 PDX). Tumor growth was observed 6 months after implantation. Passaging of tumors was performed by implanting tumor fragments of 3 x 3 x 3 mm subcutaneously into male NSG mice. To generate a castrate CP50 PDX line, tumors that had been passaged 4 times were castrated when they reached 300 to 400 mm³ and were harvested when they reached a size of around 1200 mm³. Subsequent passages were done in castrated mice. Individual CP50 PDX experiments are detailed in **Supplementary Methods**. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986.

Patient derived organoid (PDO) and patient derived xenograft-organoid (PDX-O) development

PDO and CP50 PDX-O (from CP50 PDX) were generated and maintained as

previously described (42). PDOs were successfully generated from 22 of 36 (61%) metastatic CRPC biopsies between October 2015 and April 2016; nine of which were available for growth experiments. Briefly, fresh metastatic CRPC biopsies or CP50 PDX tissue were washed in phosphate-buffered saline (PBS) supplemented with 0.5% Bovine Serum Albumin, 10 μ M ROCK inhibitor (AbMole) and subsequently minced into small pieces followed by collagenase II digestion (1:250 dilution) (ThermoFisher Scientific) for 30 to 45 minutes at 37°C. Digested biopsies were then washed in PBS supplemented as above and crude cell suspension was further separated into single cells using a 20 μ m cell strainer (BD biosciences). The resulting single cells were collected by centrifugation and suspended in organoid medium before mixing with growth factor depleted matrigel (Corning). For PDO and CP50 PDX-O growth experiments, the mixture was plated in 5 μ l drops in 96 well optical plates. Drug treatment was started 24 hours after seeding and growth was determined using CellTiter-Glo (Promega) after 7 days of treatment. For CP50 PDX-O RNA analysis, the mixture was plated in 30 μ l drops in 24-well tissue culture plates. Drug treatment was started 24 hours after seeding and organoids were harvested in cold PBS after 48 hours of treatment. RNA extraction was performed using the Quick-RNA Mini Prep kit (Zymo Research) according to the manufacturer's protocol and RNA analysis performed as described above.

Patients and tissue samples

Patients were identified from a population of men with CRPC treated at the Royal Marsden NHS Foundation Trust. All patients had given written informed consent and were enrolled in institutional protocols approved by the Royal Marsden NHS Foundation Trust Hospital (London, UK) ethics review committee (reference no. 04/Q0801/60). Human biological samples were sourced ethically and their research use was in accord with the terms of the informed consent provided. Patients and tissue samples identified can be found in **Supplementary Methods**.

Tissue analysis

Immunohistochemistry (IHC) for BRD4, AR-V7 and full length androgen receptor (AR-FL) was performed on patient samples and CP50 PDX as previously described (**Supplementary Methods**) (25, 26, 43).

Statistical analysis

All statistical analyses were performed using Stata v13.1 or GraphPad Prism v6 and are indicated within all figures and tables. Detailed methods for clinical statistical analysis can be found in **Supplementary Methods**.

3. Results

3.1 BRD4 protein expression at diagnosis associates with prostate cancer patient overall survival and increases further as patients develop castration resistant disease.

To investigate the clinical significance of nuclear BRD4 expression in PC we evaluated tumor samples from 53 patients, 15 with only diagnostic (archival) hormone sensitive PC (HSPC) biopsies and 38 patients with matched HSPC and CRPC biopsies (**Supplementary Figure S1**). BRD4 antibody specificity for immunohistochemistry (IHC) was confirmed using BRD4 knockdown and overexpression in LNCaP95 cells (**Supplementary Figure S2**). H-scores (HS) were determined by IHC for nuclear BRD4 expression in all patient biopsies (**Figure 1A**); median nuclear BRD4 expression at diagnosis in 53 HSPC biopsies was 100; (interquartile range [IQR]: 100-150) with BRD4 expression (continuous variable; per 100 HS) being significantly associated with time to castration resistance (TTCRPC) (HR 2.16; 95% CI 1.25-3.73; $p=0.006$) and overall survival (OS) (HR 2.23; 95% CI 1.23-4.02; $p=0.008$). Furthermore, patients with high nuclear BRD4 expression (HS \geq 100; $n=41$) had a significantly shorter median TTCRPC (1.8 vs 9.1 months; HR 4.75; 95% CI 2.11-10.68; $p<0.001$) and OS (5.5 vs 12.6 months; HR 3.25; 95% CI 1.50-7.01; $p<0.001$) compared to patients with low nuclear BRD4 expression (HS < 100; $n=12$) (**Figure 1B-C**). Patients with low BRD4 expression at diagnosis had lower incidence of lymph node involvement ($p=0.006$) and fewer presented with distant metastasis, although not statistically significant ($p=0.12$); and were more likely to receive curative treatment ($p=0.002$) (**Supplementary Table 6**). Thirty-eight patients with matched HSPC and CRPC biopsies were used to investigate the clinical significance of nuclear BRD4 expression as patients developed CRPC. Nuclear BRD4 expression increased significantly ($p=0.008$) as patients progressed from HSPC (median; IQR: HS 100; 100-170) to CRPC (HS 150; 110-200) (**Figure 1D**). Nuclear BRD4 expression (continuous variable; per 100 HS) in CRPC biopsies was not significantly associated with OS (HR 0.63, 95% CI 0.32-1.24; $p=0.18$). Although SPOP mutant PC have been associated with increased BRD4 protein expression, we found no clear association between SPOP mutations (4 of 39 cases with SPOP status) and BRD4 protein expression (**Supplementary Figure S3**) (31, 32). Taken together, these data suggest that higher nuclear BRD4 expression at diagnosis, but not CRPC, is associated with poorer patient outcome. In addition, nuclear BRD4 expression increases as patients progress from HSPC to CRPC, suggesting a role in disease progression and treatment resistance.

3.2 BET proteins associate with androgen receptor activity in castration resistant prostate cancer

In light of BRD4 protein levels increasing as patients develop CRPC, and its association with AR signaling, we next determined whether BRD4 and other BET family members associated with AR activity in CRPC patient samples (29, 30, 34). AR activity score was determined using two previously described gene expression signatures (**Supplementary Table 5**) (5, 41). Analysis of RNA-sequencing (RNA-seq) data obtained from 122 CRPC biopsies demonstrated BRD2 to be most highly expressed in CRPC compared to BRD3, BRD4 and C-MYC, a downstream target of BET proteins (**Figure 1E**) (44). BRD2, BRD3, BRD4 and, to a lesser extent, C-MYC

RNA expression all significantly correlated with AR activity score in CRPC biopsies (all $p < 0.001$) (**Figure 1F-I**). B2M, a frequently used housekeeping control gene, did not associate with AR activity score ($p = 0.6$) (**Figure 1J**). Taken together these results indicate that BRD2, BRD3 and BRD4 are expressed and associate with AR activity in CRPC patients.

3.3 I-BET151 downregulates AR-V7 and inhibits AR signaling in prostate cancer cell lines

BET inhibitors have been reported to regulate full length AR (AR-FL) signaling, and expression of AR splice variants (including AR-V7) implicated in CRPC development and resistance to current therapies (14, 15, 22-26, 29, 30, 34). To investigate this further, we determined the effect of I-BET151, a potent inhibitor of BRD2, BRD3 and BRD4, on AR-FL and AR-V7 expression, and AR signaling in BRD4 and AR-V7 expressing prostate cancer cell lines (**Supplementary Figure S4**) (45). LNCaP95 (androgen independent and enzalutamide resistant), 22Rv1 (androgen independent) and VCaP (androgen dependent) cells were treated with increasing concentrations of I-BET151 for 48 hours prior to analysis by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. I-BET151 significantly reduced AR-V7 and C-MYC expression with only higher concentrations of I-BET151 having limited impact on AR-FL levels in LNCaP95 and VCaP cells (**Figure 2A and C**). Furthermore, I-BET151 treatment significantly reduced expression of AR regulated transcripts (PSA and TMPRSS2) (**Figure 2B and D**). Interestingly, I-BET151 did not significantly impact AR-V7 or AR-FL expression in 22Rv1 cells but still reduced C-MYC expression and inhibited AR signaling (**Figure 2E and F**). This may, at least in part, be due to the association of a genomic rearrangement with abrogated AR generation in 22Rv1 cells and suggests that I-BET151 mediated AR-V7 regulation may not be through transcriptional regulation alone (46). A second BET inhibitor, JQ1, was used to confirm these data (**Supplementary Figure S5**). Furthermore, we investigated whether I-BET151 treatment was associated with a change in drug target expression. BRD2, BRD3 and BRD4 (VCaP cells), and BRD2 (LNCaP95 cells), RNA levels increased in response to I-BET151 treatment (**Supplementary Figure S6A and B**). In contrast, I-BET151 exposure led to decreased BRD3 and BRD4 RNA expression in LNCaP95, and no significant change in BRD2, BRD3 and BRD4 RNA expression in 22Rv1 (**Supplementary Figure S6A and C**). In summary, these data suggest, as with JQ1 and other BET inhibitors, that I-BET151 regulates AR signaling in androgen dependent VCaP cells but also in androgen independent LNCaP95 and 22Rv1 cells. However, the ability of I-BET151 to regulate AR-V7 expression seems specific to LNCaP95 and VCaP cells, which have not been described to have a genomic rearrangement of AR.

3.4 BRD2, BRD3 and BRD4 knockdown recapitulates AR-V7 mediated regulation of I-BET151 in a C-MYC independent manner

I-BET151 is a potent inhibitor of BRD2, BRD3 and BRD4 activity, and is known to have downstream inhibitory effects on C-MYC expression (44, 45). Having shown that BRD2, BRD3, BRD4 and C-MYC are expressed in CRPC biopsies, and correlate with AR transcriptional activity, we proceeded to investigate which BET family members were critical for the AR-V7 mediated effects of I-BET151 in PC cells. We determined the effect of individual (BRD2 or BRD3 or BRD4) and combined BET

protein knockdown by siRNA for 72 hours on AR-FL and AR-V7 expression, C-MYC expression and AR signaling in these cell lines (**Figure 3A-F; Supplementary Figure S7**). In both LNCaP95 (**Figure 3A-C**) and VCaP (**Figure 3D-F**) cells, knockdown of BRD2, BRD3 and BRD4 individually, or in combination, did not reduce AR-FL expression significantly. Combined knockdown of BRD2, BRD3 and BRD4, to a greater extent than BRD4 knockdown alone, reduced AR-V7 expression in both cell line models (**Figure 3A-F**). Consistent with this, treatment of LNCaP95 and VCaP cells with I-BET151 reduced AR-V7 expression (**Figure 3A and C; 3D and F**). Interestingly, I-BET151 treatment consistently reduced PSA expression in both VCaP and LNCaP95 cells whilst BET protein knockdown had no significant effect (**Figure 3C and F**). In contrast, TMPRSS2 expression was not consistently reduced by I-BET151 (as shown previously; **Figure 2B and D**). To determine whether these I-BET151 effects were through a C-MYC dependent mechanism, we analyzed C-MYC knockdown in LNCaP95 and VCaP cells (**Figure 3A and B; 3D and E**). As expected, I-BET151 treatment, and combined BRD2, BRD3 and BRD4 protein knockdown, decreased C-MYC expression. Interestingly, however, knockdown of C-MYC alone had no significant effect on AR and AR-V7 but significantly increased PSA and TMPRSS2 expression (**Figure 3A-F**). Taken together, these data demonstrate that whilst BRD4 knockdown alone is able to regulate AR-V7 expression and AR signaling, independent of C-MYC activity, the results are more pronounced when BRD2, BRD3 and BRD4 are knocked down together, consistent with I-BET151 targeting these BET proteins and functional redundancy between family members.

3.5 I-BET151 inhibits alternative splicing in prostate cancer cells

To further understand the mechanism of I-BET151 mediated AR-V7 regulation in CRPC cells we determined its effects on gene expression and associated cellular pathways. LNCaP95 cells were treated with I-BET151 (0.5 and 2.0 μM) for 48 hours and RNA-seq analysis was performed. Prior to analyzing these data, I-BET151 regulation of AR-FL, AR-V7, C-MYC and PSA expression was confirmed (as an experimental control; **Supplementary Figure S8**). Consistent with previous reports, I-BET151 regulated genes involved in chromatin remodeling and AR signaling (**Supplementary Table 7**). In addition, I-BET151 regulated genes whose proteins are implicated in the spliceosome and critical to RNA processing, suggesting that it regulates AR-V7 expression through modulation of alternative splicing. To further support this hypothesis, we demonstrated that treatment with I-BET151 led to a dose dependent change in alternative splicing events (**Figure 4A**). Treatment with 2.0 μM and 0.5 μM I-BET151 led to significant changes (determined by normalized reads count difference >2.0 or <2.0 and false discovery rate <0.05) in 137 alternative splicing events (involving 119 genes) and 47 alternative splicing events (involving 41 genes) respectively (**Supplementary Table 8**). In both cases, the majority of the significant changes in alternative splicing events (70% and 64%; 2.0 μM and 0.5 μM) decreased with I-BET151 treatment (**Supplementary Table 8**). Furthermore, RNA-seq analysis confirmed that I-BET151 reduced AR-V7 cryptic exon expression (**Figure 4B**) and reduced alternative splicing events in LNCaP95 cells (**Figure 4C**). These data suggest that I-BET151 mediated regulation of AR-V7 expression is through inhibition of alternative splicing events in PC.

3.6 Regulation of BET protein activity and C-MYC expression by I-BET151 is critical for prostate cancer growth inhibition

BET inhibitors have been shown to inhibit the growth of AR dependent and independent PC models (29, 30, 33-35). Next we determined the specific target of I-BET critical for PC cell growth inhibition. I-BET151 inhibited the growth of LNCaP95 and VCaP cells in a dose dependent manner (**Figure 5C and F**). BRD4 knockdown alone, and BRD2, BRD3 and BRD4 knockdown in combination inhibited the growth of LNCaP95 and VCaP cells (**Figure 5A and B; 5D and E; Supplementary Figure S9**). Moreover, I-BET151 treatment synergized (LNCaP95 $p=0.01$; VCaP $p=0.002$) with combined BRD2, BRD3 and BRD4 knockdown to further decrease PC cell growth (**Figure 5B and E**). Having confirmed that I-BET151 inhibition of AR signaling and downregulation of AR-V7 expression was independent of C-MYC, we next identified that C-MYC knockdown was sufficient to inhibit LNCaP95 and VCaP cell growth (**Figure 5A and C**). As previously described, these data are consistent with BET inhibitor regulation of C-MYC expression being important for PC cell growth inhibition (33).

3.7 I-BET151 regulates AR-V7 and C-MYC expression, and inhibits AR signaling and proliferation in CRPC patient derived models

We and others have shown that BETi regulate the growth of PC cell line models *in vitro* (**Figure 5C and F**) (29, 30, 33-35). We sought to determine if I-BET151 inhibited the growth of nine patient derived organoids (PDOs) derived from metastatic biopsies (5 lymph nodes and 4 bone marrow trephines) from patients with advanced CRPC who had confirmed AR aberrations (2.4 treatments: mean standard treatments for CRPC) (**Supplementary Figure S10; Supplementary Table 9 and 10**). Growth of three out of nine (33.3%) PDOs were inhibited by >50% with I-BET151 (**Figure 6A**). There were no significant differences between clinical and molecular characteristics (including SPOP status and BRD4 protein expression) of patients that these PDOs were derived from (**Supplementary Figure S10; Supplementary Table 9 and 10**). To allow further investigation of I-BET151 in patient derived models we developed a patient derived xenograft (PDX; CP50) that provided material for patient derived xenograft-organoids (PDX-O) (**Supplementary Figure S11A**). CP50 was derived from the lymph node biopsy of a CRPC patient that had progressed through all standard of care treatments (**Supplementary Figure S11B**). Whole exome sequencing of a lymph node biopsy (performed 6 months prior to the PDX parental biopsy) from the same patient and CP50 PDX confirmed similar copy number changes including gain in chromosome 8 (MYC locus) and AR amplification but no AR or SPOP mutations (**Supplementary Figure S11C**). Treatment of CP50 PDX-O with I-BET151 downregulated AR-V7 and C-MYC expression, and inhibited PDX-O growth (**Figure 6B and C**). In contrast, enzalutamide did not reduce PDX-O growth, consistent with its parental biopsy being from a CRPC patient who had developed resistance to enzalutamide (**Figure 6B and C**). AR-V7 expression increases as PC patients progress to CRPC and develop treatment resistance (14, 15, 22-26). Consistent with this, castration of CP50 PDX for 14 days significantly increased AR-V7 expression, with a less pronounced impact on AR-FL, C-MYC expression and AR signaling (**Figure 6D and E; Supplementary Figure S12A; Supplementary Figure S13A and B**). Having shown I-BET151 reduced AR-V7 expression and alternative splicing events in PC cell lines, we

explored whether I-BET151 could repress AR-V7 generation in this model. I-BET151 significantly reduced AR-V7 mRNA expression and reduced AR-V7 protein expression (**Figure 6D and E; Supplementary Figure S12A; Supplementary Figure S13A and B**). I-BET151 also reduced C-MYC, PSA and (to a lesser degree) TMPRSS2. Finally, we determined the effect of I-BET151 and enzalutamide in CP50 PDX grown in castrate mice for 40 days (**Supplementary Figure S12B**). I-BET151, but not enzalutamide, significantly reduced tumor growth when compared to vehicle (**Figure 6F**), and significantly prolonged survival (**Figure 6G**). These data indicate that I-BET151 reduces the expression of AR-V7 and C-MYC, thereby inhibiting AR signaling and tumor growth in advanced, treatment resistance, CRPC.

4. Discussion

Advanced PC responds to ADT, but invariably progresses to lethal CRPC with incontrovertible evidence of ongoing AR signaling. This has led to advances in antiandrogen therapy, with the successful development of abiraterone and enzalutamide (9-13). Despite these advances, primary resistance is common and secondary resistance on treatment inevitable in CRPC, in part due to expression of constitutively active AR splice variants that evade current antiandrogen treatment strategies (14, 15, 21-26). One promising strategy undergoing clinical evaluation in CRPC is targeting BET family proteins (ClinicalTrials.gov identifier: NCT02711956, NCT03150056). Several BETi, and PROTAC induced BET protein degraders, have shown promising efficacy in preclinical models of CRPC, regulating AR signaling and AR-V7 expression (29, 30, 33-36). Although the mechanism by which BET inhibition regulates AR signaling is well described, the ability to regulate expression of AR-V7 is not yet understood (29, 30, 34-36).

BET family proteins are expressed in PC (35). Here we show that BET family proteins associate with AR activity in clinical samples and that BRD4 protein expression increases as castration resistant disease develops. In addition, we show that lower BRD4 protein expression is associated with improved patient outcome at diagnosis but not CRPC. This is likely to reflect the demonstration that patients with lower BRD4 expression are more likely to present with local disease (lymph node negative, no distant disease) and receive radical treatment; an observation that warrants further investigation. SPOP mutant cancers have been identified as a subgroup of prostate cancer with increased BRD4 expression (31, 32). Although we were unable to confirm these findings, this is likely explained by the small numbers (4 cases) of SPOP mutant cases and different patient population. These patient derived data support preclinical studies demonstrating BRD4 to be a critical coregulator of AR activity and identifying its potential role in treatment resistance (29, 30, 35, 36). BETi and PROTAC-induced BET protein degradation have been reported to regulate AR signaling and more recently AR-V7 expression (29, 30, 35, 36). Consistent with this, we demonstrate that the BET inhibitors, I-BET151 and JQ1, inhibit AR signaling and reduce AR-V7 expression in VCaP and LNCaP95 cells. Interestingly, both inhibitors had no effect on AR-V7 expression in 22Rv1 cells, probably due to its generation in this model, in part, resulting from AR genomic rearrangements, as opposed to alternative splicing (15, 46, 47). These data suggest that I-BET151 mediated AR-V7 regulation may be through the inhibition of alternative splicing, and indicate that patient selection for treatment with these agents should consider AR-V7 positive but AR genomic rearrangement negative tumors (30).

BETi abrogation of AR signaling is well described (29). However, the mechanism by which BETi regulate AR-V7 expression has not been fully explored (30, 36). Importantly, our study demonstrates that simultaneous knockdown of BRD2, BRD3 and BRD4 is more effective at downregulating AR-V7 expression than individual BET family protein knockdown, suggesting a degree of functional redundancy between BET family proteins (45). As expected combined BET family protein knockdown regulates C-MYC expression. However, I-BET151 mediated C-MYC regulation was not needed for abrogation of AR signaling and reduced AR-V7 expression; indeed C-MYC knockdown alone had no effect on AR-V7 expression and increased AR signaling. These data indicate that in BETi early clinical trials the pharmacodynamic

confirmation of downregulated AR signaling, C-MYC and AR-V7 in CRPC serial biopsies should be demonstrated to support dose/schedule selection.

AR-V7 is transcribed from alternatively spliced transcripts of AR mRNA (48). Although not fully understood, BETi have been shown to regulate expression of splicing factors possibly key to AR-V7 generation (30, 49). Critically, we show that I-BET151 regulates the expression of previously undescribed genes associated with the spliceosome complex and genes important for RNA processing. Furthermore, we present for the first time that I-BET151 regulates AR-V7 transcript expression and alternative splicing events in prostate cancer cells. Taken together, these data provide further evidence that BET inhibitors inhibit AR-V7 generation through regulation of RNA processing and in particular alternative splicing (30, 49). Aberrant splicing has been implicated in the pathogenesis of other (beyond PC) malignant and non-malignant disease processes (50). In light of our findings, further studies are now warranted to determine whether BET inhibitors provide a novel therapeutic approach to inhibit RNA processing and abrogate development/progression of these diseases.

I-BET151 inhibited the growth of PC cell lines. Interestingly, I-BET151 treatment modulated BRD2, BRD3 and BRD4 expression in PC cell lines which may provide early suggestions toward a mechanism of resistance to BETi therapy. Consistent with this; I-BET151 treatment alongside combined BET protein knockdown (preventing upregulation) was more potent than I-BET151 treatment alone. Early clinical trials will need to quickly establish whether these data are recapitulated in CRPC patients supporting its role as a resistance mechanism. In addition to PC cell lines, I-BET151 inhibited the growth of PDOs derived from fresh metastatic CRPC patients who had progressed despite abiraterone and/or enzalutamide treatment. These data suggest that BET inhibitors may provide therapeutic approaches to overcome AR resistant mechanisms in CRPC including AR amplification and AR-V7 expression that associate with worse outcome and resistance to abiraterone and/or enzalutamide (6, 14, 15, 22-26, 47). To further investigate this, we developed a PDX-O and PDX (CP50) from a patient who had progressed on both abiraterone and enzalutamide with tumor AR amplification and AR-V7 protein expression. I-BET151 inhibited the growth of this CP50 PDX-O and downregulated AR-V7 expression. Consistent with the presence of AR amplification and AR-V7 expression, enzalutamide had no growth inhibitory effects in this model. These data support the study of hypothesis-testing clinical trials with BET inhibitors to overcome persistent AR signaling in CRPC (29, 30, 36)

Studies have shown that AR-V7 expression increases as patients develop resistance to endocrine therapies (15, 21-26). Consistent with this apparent induction of AR-V7 in response to ADT, our CP50 PDX model demonstrated an increase in AR-V7 expression upon castration that was blocked by I-BET151. These data suggest that I-BET151 can reduce AR-V7 expression, consistent with previous studies of BET inhibitors and PROTAC-induced BET protein degraders in cell based models (30, 36). Finally, I-BET151 inhibited the growth of castrate CP50 PDX which is enzalutamide/abiraterone resistant supporting studies associating AR aberrations with these drug resistance phenotypes. Although the studies were performed in a single PDX-O/PDX model, it represents a common PC patient population that currently have no standard therapies with proven efficacy and represents an area of

unmet clinical need. These data provide further evidence that BET inhibitors may have clinical utility in this patient population (15, 21-26).

In conclusion, our study supports the clinical interrogation of BET inhibitors in patients with CRPC resistant to abiraterone and/or enzalutamide. It is likely with the repositioning of abiraterone to treating first line, advanced HSPC, that as patients develop CRPC the incidence of AR-V7 positive disease will increase (9). Therefore, the development of therapies that overcome these mechanisms of persistent AR signaling is an area of urgent unmet clinical need. BET inhibitors show promise, but ongoing clinical studies will need to: (1) demonstrate their safety profile in light of the plethora of cellular processes they have been reported to regulate; (2) confirm pharmacodynamic regulation of AR signaling and AR-V7 (and other splice variants) in CRPC tissue at a safe dose and schedule not causing dose limiting thrombocytopenia; and (3) induce durable anti-cancer responses. If these clinical trials provide this data, then BET inhibitors have the potential to provide a novel therapeutic strategy to overcome AR aberrant signaling in CRPC and improve patient outcome beyond current clinically approved therapies.

5. Figure Legends

Figure 1: BET family protein expression, association with AR signaling and patient outcome in prostate cancer

(A) Representative micrographs of BRD4 detection by immunohistochemistry in matched hormone sensitive prostate cancer (HSPC) and castration resistant prostate cancer (CRPC) biopsies (magnification 200x; scale bar 50 μ m). **(B-C)** Kaplan-Meier curves of time to CRPC (TTCRPC) (B) and overall survival (OS) (C) from diagnosis for nuclear BRD4 expression at diagnosis (HSPC) by H-score < 100 (green; n=12) or \geq 100 (orange, n=41). Hazard ratio (HR) with 95% confidence intervals and p-values for univariate cox survival model are shown. **(D)** Expression (H-score) of nuclear BRD4 expression in 38 matched patient samples at HSPC (grey) and CRPC (red). Median H-score and interquartile range is shown. p-value (*p=<0.05, **p=<0.01, ***p=<0.001) was calculated using Wilcoxon matched-pair signed rank test. **(E)** BRD2, BRD3, BRD4 and C-MYC RNA expression in fragments per kilobase of transcript per million mapped reads (FPKM) for 122 CRPC transcriptomes are shown. Median FPKM and interquartile range is shown. **(F-J)** Association between BRD2 (F), BRD3 (G), BRD4 (H), C-MYC (I) and B2M (J) expression levels and androgen receptor AR signaling score (derived from 43 AR regulated genes; **see supplementary table 6**) from 122 CRPC transcriptomes are shown. p-values were calculated using linear regression analysis.

Figure 2: I-BET151 treatment downregulates AR-V7 and AR signaling in androgen dependent and independent prostate cancer cell lines

LNCaP95 **(A-B)**, VCaP **(C-D)** and 22Rv1 **(E-F)** were treated with vehicle (DMSO 0.1%) or various concentrations of I-BET151 (0.1 μ M, 0.5 μ M, 1.0 μ M, 2.5 μ M and 5.0 μ M) for 48 hours. The effect of I-BET151 treatment on androgen receptor full length (AR-FL), androgen receptor variant 7 (AR-V7) and C-MYC protein expression (A, C, E) and AR-FL, AR-V7, C-MYC, prostate specific antigen (PSA) and TMPRSS2 RNA expression was determined (B, D, F). Single representative western blot shown from three separate experiments. Mean RNA expression (normalized to B2M and vehicle; defined as 1.0) with standard deviation from three individual experiments is shown (unless otherwise stated). p-values (*p=<0.05, **p=<0.01, ***p=<0.001) were calculated for each I-BET151 dose compared to vehicle (DMSO 0.1%) using unpaired student's t-test.

Figure 3: BET family protein knockdown recapitulates I-BET151 effects on AR-V7 expression and AR signaling independent of C-MYC expression

LNCaP95 **(A-C)** and VCaP **(D-F)** were transfected with 50 nM control, BRD2, BRD3, BRD4 or C-MYC siRNA (LNCaP95 A and B; VCaP D and E); or 150 nM control or 150 nM (50 nM each) BRD2, BRD3 and BRD4 siRNA with I-BET151 (0.5 μ M) or without (vehicle; DMSO 0.1%) for 72 hours (LNCaP95 A and C; VCaP D and F). The effect of each condition on AR-FL, AR-V7, C-MYC and PSA protein expression (LNCaP95 A; VCaP D) and AR-FL, AR-V7, C-MYC, PSA and TMPRSS2 RNA expression was determined (LNCaP95 B-C; VCaP E-F). Single representative western blot shown from three separate experiments. Mean RNA expression (normalized to B2M and control siRNA at equivalent concentration; defined as 1.0)

with standard deviation from three individual experiments is shown. p-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were calculated for each condition compared to control siRNA (at equivalent concentration) using unpaired student's t-test.

Figure 4: I-BET151 decreases AR-V7 expression by decreasing alternative splicing events

LNCaP95 cells were treated with vehicle (DMSO 0.1%) or I-BET151 (0.5 μM or 2.0 μM) for 48 hours prior to RNA-seq analysis being performed (single experiment in duplicate). **(A)** Unsupervised cluster and heatmap of 137 significantly (determined by normalized reads count difference >2.0 or <2.0 and false discovery rate <0.05) changed alternative splicing events induced by I-BET151 treatment. **(B)** Sashimi plot represents reduced AR-V7 cryptic exon expression after I-BET151 treatment. Arcs representing splice junctions connect exons. The bridge number between exon 3 and cryptic exon in intron 3 is the AR-V7 expression level. **(C)** Alternative splicing events are schematically represented and histograms of alternative splicing mean differences between vehicle (blue dotted line; defined as 0.0) and I-BET151 (0.5 μM or 2.0 μM) in five categories of alternative splicing events are shown (left shift; decrease in splicing events).

Figure 5: BET family protein knockdown and I-BET151 inhibits the growth of androgen dependent and independent prostate cancer cell lines

LNCaP95 **(A-B)** and VCaP **(D-E)** were transfected with 50 nM control, BRD2, BRD3, BRD4 or C-MYC siRNA; or 150 nM control or 150 nM (50 nM each) BRD2, BRD3 and BRD4 siRNA with I-BET151 (0.5 μM) or without (vehicle; DMSO 0.1%) I-BET151 and growth determined after 7 days using Sulforhodamine B (SRB) colorimetric assay. LNCaP95 **(C)** and VCaP **(F)** were treated with vehicle (DMSO 0.1%) or various concentrations of I-BET151 (0.1 μM , 0.5 μM , 1.0 μM , 2.5 μM and 5.0 μM) and growth determined after 7 days by SRB colorimetric assay. For all experiments (A-F), mean growth (normalized to control siRNA at same concentration or vehicle) with standard deviation from four individual experiments is shown. p-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were calculated for each condition compared to control siRNA (at same concentration) or vehicle using unpaired student's t-test.

Figure 6: I-BET151 decreases AR-V7 expression and inhibits growth in prostate cancer patient derived models

(A) Nine patient derived organoids (PDO) were developed from metastatic biopsies and treated with vehicle (DMSO 0.1%) or I-BET151 (0.5 μM or 5.0 μM) and growth determined after 7 days using CellTiter-Glo® assay. Mean growth (normalized to vehicle; defined as 1.0) with standard deviation from one experiment performed in duplicate is shown (unless otherwise stated). *Low organoid number. **(B)** Patient derived xenograft-organoid (PDX-O) from patient derived xenograft (PDX) CP50 was treated with vehicle (DMSO 0.1%) or 1.0 μM I-BET151 or 10 μM enzalutamide for 7 days. Mean growth (normalized to vehicle; defined as 1.0) with standard deviations from one experiment performed in triplicate is shown. p-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were calculated for each condition compared to vehicle (DMSO 0.1%) using unpaired student's t-test. **(C)** AR-FL, AR-V7 and C-MYC RNA expression was

determined. Mean RNA expression (normalized to B2M and vehicle; defined as 1.0) is shown from a single experiment. **(D-E)** PDX CP50 mice were either left intact (n=2; unshaded box) or castrated (n=8; shaded box) for 7 days prior to being treated with either vehicle (2 intact mice and 4 castrate mice) or 15 mg/kg I-BET151 (4 castrate mice) for 11 days. The effect of each condition on AR-FL and AR-V7 protein expression (D) and AR-FL, AR-V7, C-MYC, PSA and TMPRSS2 RNA expression was determined (E). Mean RNA expression (normalized to B2M and vehicle-castrate; defined as 1.0) with standard deviation from the number of mice described is shown. p-values (*p=<0.05, **p=<0.01, ***p=<0.001) were calculated for each condition compared to vehicle-castrate using unpaired student t-test. **(F-G)** PDX CP50 were castrate and once tumor volume reached 300mm³ treated with either vehicle (n=6) or 15 mg/kg I-BET151 (n=7) or 10mg/kg enzalutamide (n=6) daily until reaching 300% of starting volume or 40 days treatment. Mean growth (normalized to start; defined as 100%) with standard deviation was determined for each tumor (F). p-values (ns non-significant, *p=<0.05, **p=<0.01, ***p=<0.001) were calculated using a longitudinal mixed effect model with per mouse random intercept and slope. Time to reach 300% growth was used as a surrogate endpoint for survival (G). One mouse in both the I-BET151 and enzalutamide treatment group was culled before 300% growth ([^]294% and [^]266%; as reached legal limit due to increased baseline volume) and censored for analysis. Median overall survival (OS), hazard ratio (HR) with 95% confidence intervals and p-values for univariate cox survival model are shown. Median overall survival (OS) for I-BET151 was not reached (NR) at 40 days treatment.

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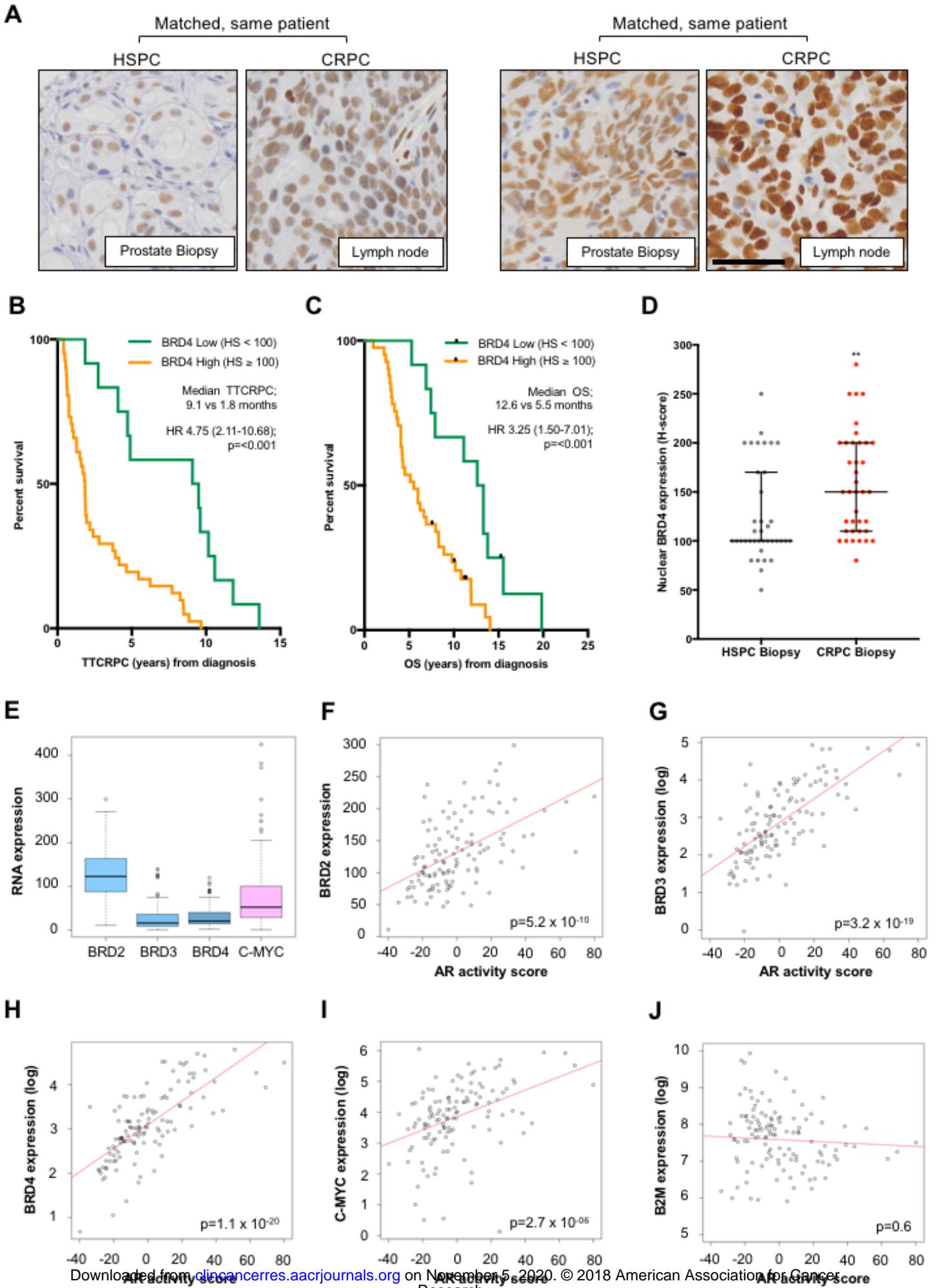
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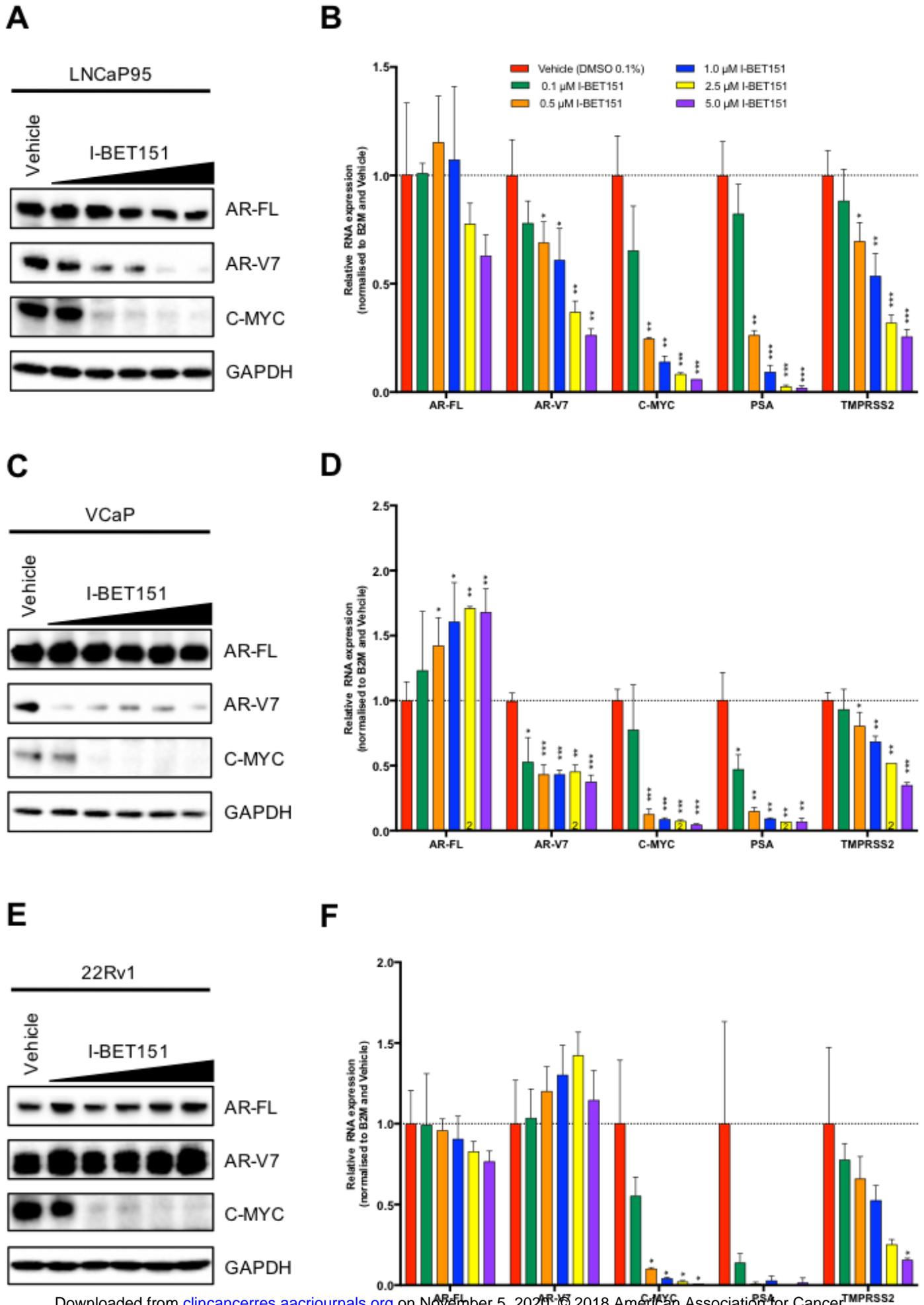
7. Competing interests

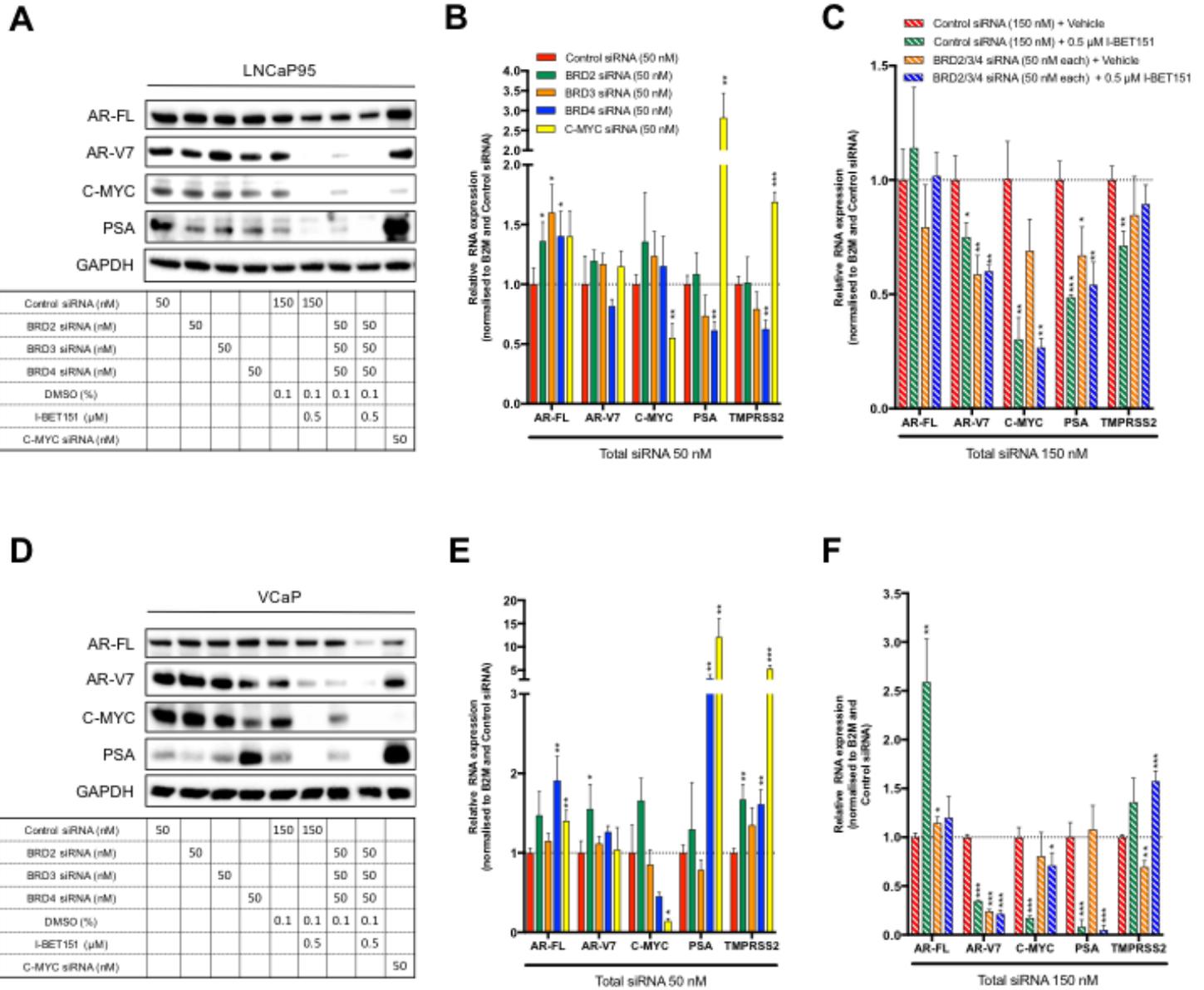
JW, AS, WY, DD, DNR, IF, VG, AN, MC, GS, MC, SS, JN, EK, JCF, AP, SC, GB, AS and JSdB are employees of The Institute of Cancer Research, which has a commercial interest in abiraterone. JSdB has served as a consultant/advisory member for Astellas Pharma, AstraZeneca, Bayer, Genmab, Genentech, GlaxoSmithKline, Janssen, Medivation, Orion Pharma, Pfizer and Sanofi.

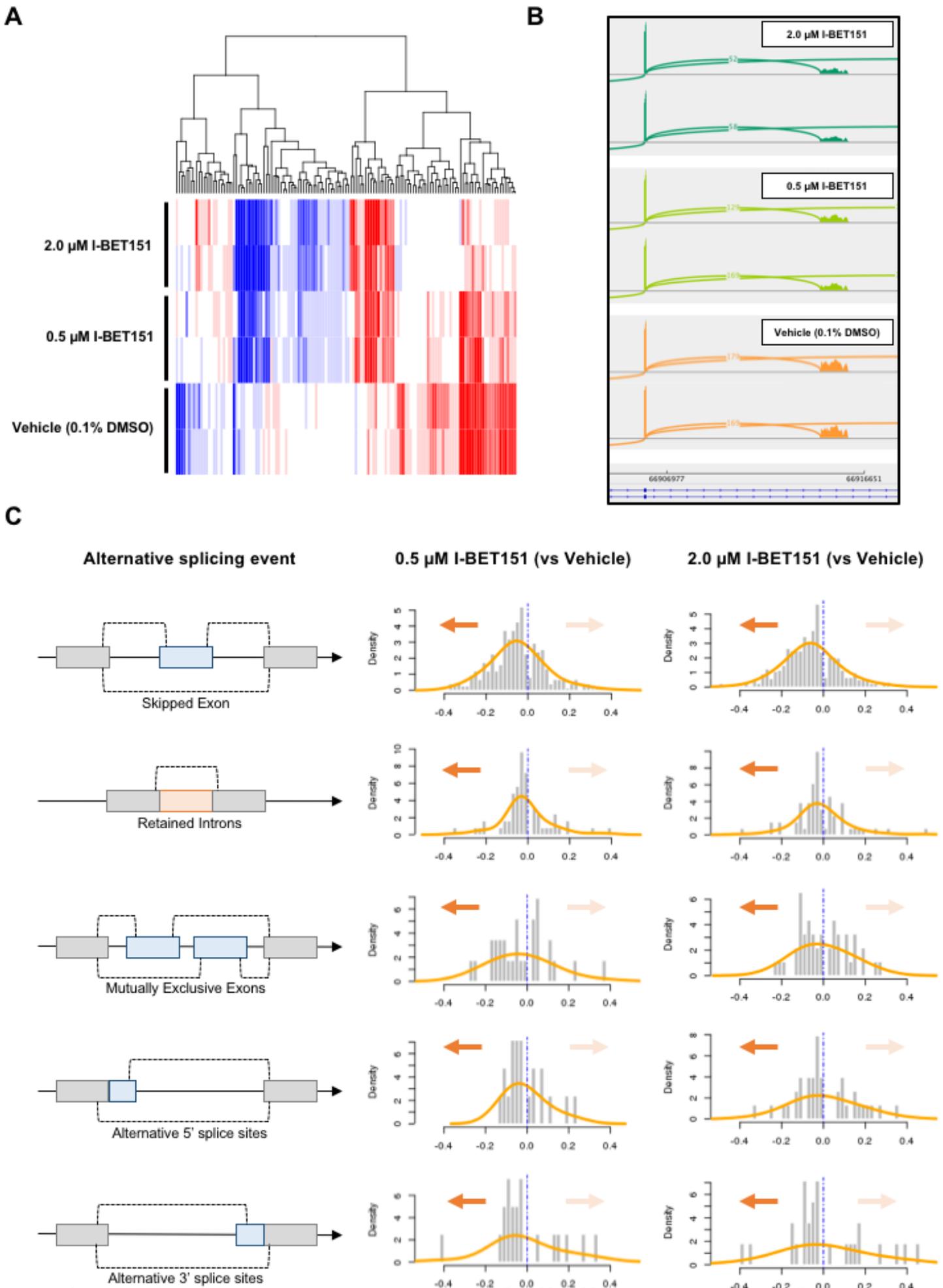
8. Funding

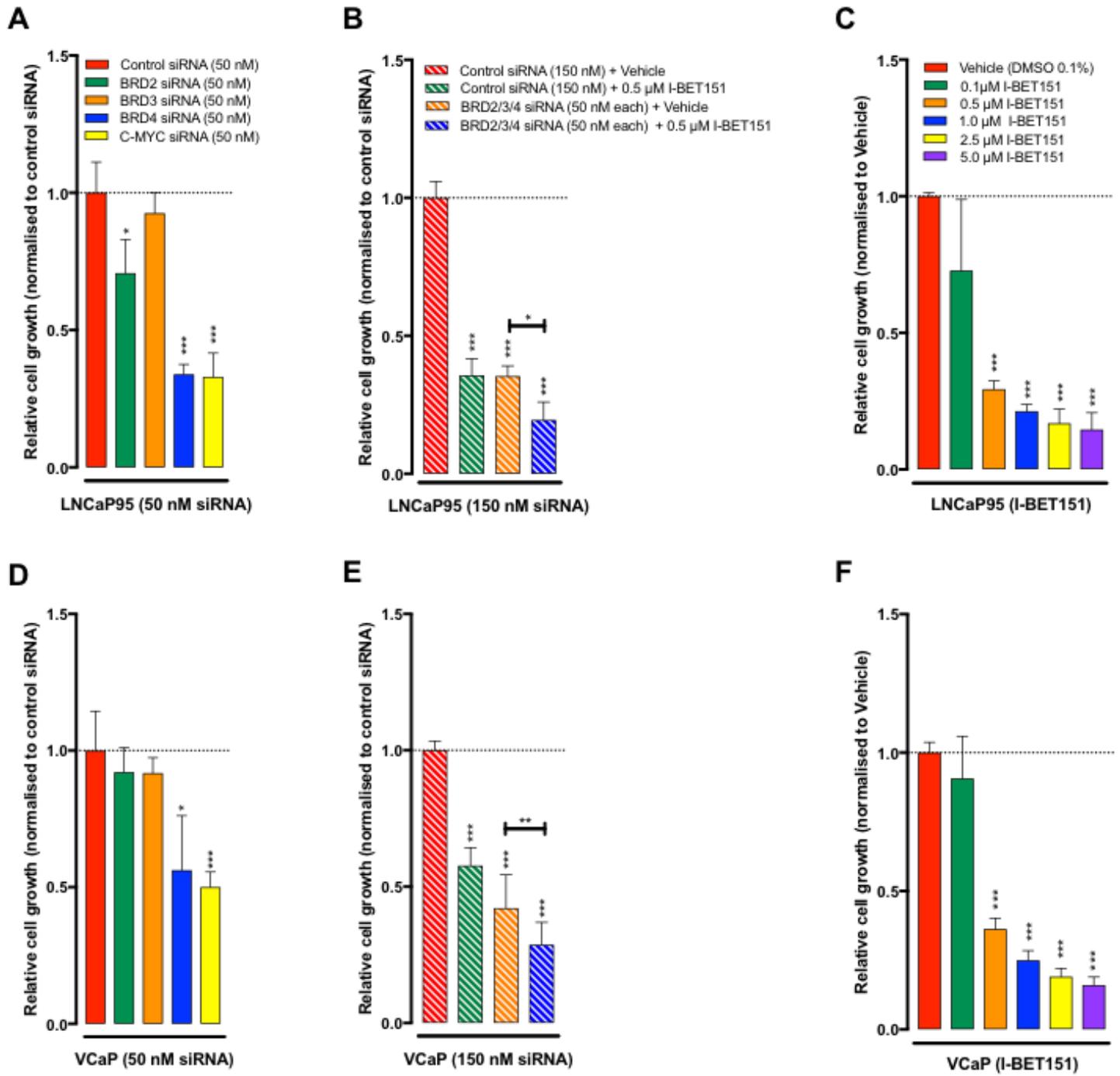
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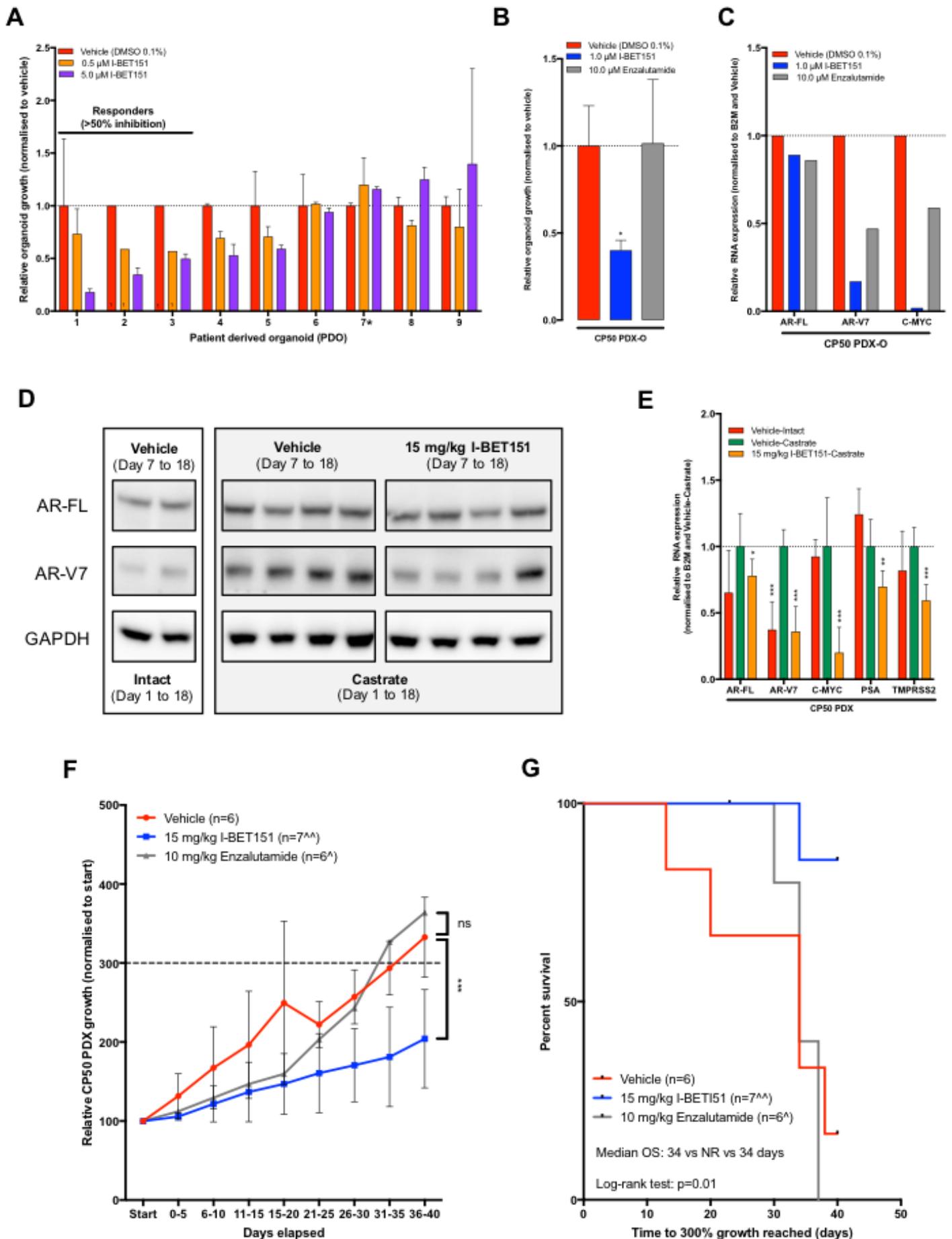












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Targeting bromodomain and extra-terminal (BET) family proteins in castration resistant prostate cancer (CRPC)

Jonathan Welti, Adam Sharp, Wei Yuan, et al.

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