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Targeting the BRD4-HOXB13 Co-regulated Transcriptional Networks with Bromodomain-Kinase Inhibitors to Suppress Metastatic Castration-Resistant Prostate Cancer

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Abstract

Resistance to Androgen receptor (AR) antagonists is a significant problem in the treatment of Castration resistant prostate cancers (CRPCs). Identification of the mechanisms by which CRPCs evade Androgen Deprivation Therapies (ADT) is critical to develop novel therapeutics. We

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uncovered that CRPCs rely on BRD4-HOXB13 epigenetic reprogramming for androgen-independent cell proliferation. Mechanistically, BRD4, a member of the BET bromodomain family epigenetically promotes HOXB13 expression. Consistently, genetic disruption or pharmacological suppression of HOXB13 mRNA and protein expression by the novel dual activity BET bromodomain-kinase inhibitors directly correlates with rapid induction of apoptosis, potent inhibition of tumor cell proliferation, inhibits cell migration and suppresses CRPC growth. Integrative analysis revealed that the BRD4-HOXB13 transcriptome comprises a proliferative gene network implicated in cell cycle progression, nucleotide metabolism and chromatin assembly. Notably, while the core HOXB13 target genes responsive to BET inhibitors (HOTBIN10) are overexpressed in metastatic cases, in ADT treated CRPC cell lines and patient derived circulating tumor cells (CTCs) they are insensitive to AR depletion or blockade. Among the HOTBIN10 genes, AURKB and MELK expression correlate with HOXB13 expression in CTCs of mCRPC patients who did not respond to Abiraterone (ABR), suggesting that AURKB inhibitors could be used additionally against high-risk HOXB13 positive metastatic PCs. Combined, our study demonstrates that BRD4-HOXB13-HOTBIN10 regulatory circuit maintains the malignant state of CRPCs and identifies a core pro-proliferative network driving ADT resistance that is targetable with potent dual activity bromodomain-kinase inhibitors.

Keywords

HOXB13; BRD4; AR; Epigenetics; Metastasis; Prostate Cancer

Introduction

Prostate cancers can be slow growing and non-life threatening or aggressive and lethal. Prognosis of castration resistant prostate cancer (CRPC) is bleak; the castration resistant forms relapse in 2–3 years despite treatments with the second-generation AR blockers, Abiraterone (ABR) or Enzalutamide (ENZ). Thus, CRPC remains a leading cause of cancer related deaths in American men and the five-year survival rate is only about 29% due to the limited therapeutic options (1, 2). Metastatic CRPC is considered an “epigenetic disease” as mutations are found at a much lower frequency in comparison with other solid tumors (3–5). Epigenetic deregulation can lead to aberrant activation of tissue specific and developmentally regulated transcription factors and may be one mechanism driving castration resistance. Thus, identification of factors driving metastasis and anti-androgen resistance is critical to target, treat the resistant forms of the disease, to improve patient outcomes.

HOXB13 is a lineage-specific homeodomain containing transcription factor predominantly expressed in the prostatic tissues. Despite the concurrent expression with AR during prostate development in mice, HOXB13 expression is largely independent of androgen (6, 7). Moreover, HOXB13 regulates AR function in a context dependent manner to promote or suppress AR transactivator function at various target genes (8). This may underlie its differential roles in regulating hormone-sensitive versus hormone refractory prostate cancers (9–11). Not only the full-length AR, HOXB13 also regulates the genomic recruitment of AR-V7, a splice variant of AR that lacks the ligand binding domain, to define the AR-V7

cistrome in CRPCs (12). Consistently, HOXB13 expression is a feature of a majority of AR positive PCs and bone mCRPCs and correlates with poor prognosis (13). Further, a rare germline mutation in *HOXB13* (G84E) was uncovered which was not only associated with an increased risk of familial and hereditary PC in different ethnic populations but male carriers develop the aggressive form of the disease with an earlier onset (14–17). Consistently, a recent study revealed poor prognosis and an early death for metastatic CRPC patients positive for HOXB13 circulating tumor cells (CTCs), following treatment with the ABR (18). Although highly correlative, it is unclear whether HOXB13 is essential for CRPC growth as well as the identity of its key effectors driving metastatic progression is unknown. Importantly, germline mutations in HOXB13 are rare; we reasoned that CRPCs may epigenetically promote deregulated expression that may underlie its role in malignancy.

A notable AR transcriptional co-regulator is the Bromodomain and Extra-Terminal (BET) domain containing protein, BRD4 (19, 20). The members of the BET family, BRD2 and BRD4 have essential functions during embryonic development and also regulate the pluripotency of embryonic stem cells (21). BRDs bind acetylated lysine residues at the N-terminus of histone H3/H4 or the non-histone proteins such as the AR and the prototype BET inhibitor JQ1 blocks this recognition to suppress the expression of target genes, such as c-MYC and PSA (19, 22). While c-MYC expression is suppressed, it does not appear to be a major target of BRD4 inhibition in CRPCs (19, 20). We report for the first time that the BET domain protein, BRD4, binds the enhancer of *HOXB13* gene upregulating its expression and this BRD4-HOXB13 epigenetic axis activates AR independent cell cycle programs to promote CRPC proliferation. Combined, our study uncovers a conserved BRD4-HOXB13 transcriptomic network in mCRPCs that promotes cancer cell proliferation despite androgen deprivation and is targetable with novel small molecule bromodomain-kinase inhibitors.

Materials and Methods

Cell Culture

The human prostate cell lines 22Rv1, DU145, LNCAP, C4–2, PC3, RWPE-1 and VCAP were directly purchased from American Type Culture Collection (ATCC) that have been authenticated by Short Tandem Repeat (STR) Profiling and grown as recommended by ATCC. All cell lines in the current study were used within 3 months or ~6–8 passages upon receipt and replenished from frozen stocks. C4–2B was grown as described earlier (2). HOXB13 CRISPR/Cas9 (GFP expressing) gene editing and HOXB13 HDR plasmid (RFP expressing) constructs were purchased from Santa Cruz. Cultures are routinely tested for mycoplasma contamination with a sensitive PCR based screening using the PCR Mycoplasma Test Kit I/C from Promokine once in two months (PK-CA91–1048).

Antibodies, Compounds, siRNAs and Primers

Anti-Actin (Sigma-Aldrich, A2228), Anti-Vinculin (Sigma-Aldrich, V9131), Histone H3K27ac pAb (Active Motif, 39133), Anti-Acetyl-Histone H4K16 (EMD Millipore, 07–329), Anti-Acetyl-H4K12 (EMD Millipore, 07–595), H4K5Ac (CST 8647), H4K8Ac (Active Motif), H3K37me3 (CST 9733S), H3K4me1 (Active Motif, 39398), H3K4me3 (CST 9727S), RNA Pol II (Active Motif, 101307), Normal Rabbit Ig (CST, 2729), HoxB13

(H-80), Santa Cruz Biotechnology sc-66923, HoxB13 (H-9), sc-28333, TAF1 Rab mAb (CST, D6J8B), AR (C-19), sc-815, AR (N-20), sc-816, BRD4 Antibody Bethyl Laboratories A301-985A50, Cleaved PARP (Asp214) (D64E10) XP Rabbit mAb, CST, 5625, c-MYC (CST, D84C12), IgG (Active Motif 101226), AR ChIP Ab (Abcam, ab3509) were purchased from commercial sources. MA4-022-1 (Compound 3), MA4-022-2 (Compound 4), SG3-179 (Compound 5), MA3-068-1 (Compound 1) and MA6-082 (a tetherable analog of MA4-022-1) synthesis and structures are described (23). Mass spectrometry and HNMR were performed to confirm the purity of each of the above compounds. The following compounds were all purchased from Selleck Chemicals and structures for the non-FDA approved compounds are shown in Supplementary table S1; Enzalutamide (S1250), JQ1 (S1047), Barasertib (AZD1152-HQPA)(S1147) Abiraterone acetate (S2246), GSK-126 (S7061), Fedratinib (S2736), Ruxolitinib (S1378) and iBET-762 (S7189). siRNAs were purchased from Santacruz; Human BRD4 siRNA (SC43639), BRD3 siRNA (SC60284), BRD2 siRNA (SC60282), BRDT siRNA (SC60286), Control siRNA (SC37007), and AR siRNA (SC29204). HOXB13 siRNA (SR307118c from Origene) and BRD4 siRNAs were purchased from Dharmacon (Supplementary table S1).

Drug Affinity Chromatography, Mass Spectrometry and Bioinformatics Analysis

Drug affinity chromatography experiments were conducted as described (25). Briefly, MA6-082, a tetherable analog of MA4-022-1, and ampicillin were immobilized on NHS-activated Sepharose for Fast Flow resin (GE Healthcare) and blocked with ethanolamine overnight. C4-2B cells lysate containing 1 mg of protein was added to the affinity matrix for 2 hours and processed. Competition experiments were conducted by incubating total cell lysates with 20 μ M MA4-022-1 for 30 min prior to affinity chromatography.

A nanoflow ultra high-performance liquid chromatograph (RSLC, Dionex, Sunnyvale, CA) coupled to an electrospray bench top orbitrap mass spectrometer (Q-Exactive plus, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. Data was searched by Mascot (v2.4.1) using the Swiss-Prot human database. Following protein ID the data was filtered (95% minimum peptide threshold, 95% protein threshold, 10 ppm parent tolerance, 0.05 Da fragment tolerance) (0.6% peptide FDR, 3.4% protein FDR) using Scaffold 4.6.1 (Proteome Software). A maximum of two missed cleavages was allowed. The data was then exported and imported into Galaxy (26–28) for analysis with the affinity proteomics analysis tool, APOSTL (<http://apostl.moffitt.org>) (29). The data was pre-processed into inter, bait and prey files and analyzed by SAINTexpress (30) and the CRAPome (31) within APOSTL.

RNA analysis

RNA was prepared and quantified as described earlier (2). Raw data (reads) were quality controlled by FastQC algorithm. Mapping to human genome (hg38) was done by tophat2 (32). SAMTools (33) was used to select only mapped reads. Alignment files (.bam) were then imported into Partek NGS (Partek, Inc.) computing reads counts per gene and transcript and converting them to RPKM (Reads Per Kilo base per Million) data. Feature summarization step in Partek uses Expectation-Maximization (EM) approach to estimate transcript abundance. Obtained RPKM signals were further Log2 transformed. Box and

whiskers plot were used to verify that sample distributions had no outliers. After the import and QC were performed, we created phenotypic groups and defined differentially expressed genes (DEGs) between biological groups of interest using DEseq procedure in R software (34). MetaCore and NextBio systems were used for enrichment analysis to identify which GO Processes, Pathway Maps, Process Networks, Diseases and Metabolic Networks were represented in the analysis data with greater statistical significance. Heat maps and Venn diagrams were produced with Partek (Partek, Inc.) and GENE-E software from Broad Institute (<https://software.broadinstitute.org/GENE-E/>). Primers used for qRT-PCR for detection of HOTBIN10 genes, HOXB13 and Actin, as well as for ChIP analysis of BRAH1/BRAH2, and IGX1A (control primers) are listed in Supplementary tables (S2 and S3).

Moffitt Total Cancer Care (TCC) Data Set

Moffitt Information Shared Services/Collaborative Data Services Core provided the de-identified gene expression data for the 194 primary and 29 metastatic tumors (Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray [HuRSTA_2a520709.CDF]) from the Moffitt Total Cancer Care (TCC) dataset. Studies that fall under the TCC protocol have been obtained with patients' written consent. The metastatic samples were obtained from multiple sites (lung, liver, bone, lymph nodes and soft tissue), while primary samples were obtained from Anterior/Posterior Apex/mid/base of prostate. All samples passed multiple internal QC filters. All probe set expression values were normalized using IRON (35). In case of multiple probe sets, the gene expression value was represented by the probe set with the largest median intensity. GSE21034 series, MSKCC data set (36) was used for validation studies.

Principle Component Analysis (PCA)

Evince multivariate analysis software version 2.7.0 (UmBio AB, Umeå, Sweden) was used for this purpose. After PCA plots were generated using all genes in a given list, the initial gene lists were reduced to those genes with the highest weightings in order to maximize the fraction variability explained by the first principal component.

Animal Studies

All animal experimentation was performed using the standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals and conducted under a protocol approved by the University of South Florida IACUC Committee (IACUC 2095R). 4-week-old intact or castrated male SCID mice (n=10–12/group) obtained from Charles Rivers Laboratories. Mice were implanted subcutaneously with 2×10^6 C4–2B or VCaP cells suspended in 100 μ l of PBS with Matrigel (BD Biosciences) into the dorsal flank. Mice were randomized when the tumors reached 100–200 mm³, and treated with subcutaneous injections of either the vehicle (control) or MA4-022-2 or JQ1 at 50 mg/kg body weight for 5 days/week for 4 weeks. Measurements of the tumors from were made using digital calipers twice a week and tumor volumes were calculated. Tumors, blood and organs were harvested from the vehicle and treated animals after euthanasia for histopathology analysis and sampled for RNA expression profiling.

Statistical Analysis

Differences in means between individual groups were analyzed by Student's *t*-test, or one-way analysis of variance (ANOVA). ANOVA was followed by Sidak's multiple comparison tests to correct for multiple comparisons. Statistical assumptions of homogeneous variance and normality were tested using Levene's *F* test and Shapiro test, respectively. Wilcoxon-rank sum test was used to compare the groups when the assumptions were violated. All analyses were made using the Graph Pad Prism 6.0 software. Two-sided *p*-values < 0.05 were considered statistically significant.

Statistical analysis of human prostate tumor data

Statistical analyses were done using R 3.4.1. A stepwise variable selection method based on Akaike information criterion (AIC) was used to select the optimal logistic regression model. Receiver operating characteristic (ROC) curve was used to evaluate overall performance of the logistic regression model (37). Wilcoxon rank sum test was used to compare Gleason scores of two groups.

Data and Software Availability

RNA sequencing Accession numbers: GSE112298; GSE11230.

Results

BRD4 mediated epigenetic regulation of *HOXB13* gene expression is androgen independent

A finer analysis for potential BRD4 binding sites revealed three specific peaks in the *HOXB13* upstream region in Chromatin immunoprecipitation (ChIP)-sequencing data in VCaP cells (19). Of these, two of the major BRD4 binding peaks located at the 268th nucleotide position (**BRAH1**-BRD4 recruitment **At HOXB13 1**) and at the 799-nucleotide position (**BRAH2**) upstream from the *HOXB13* transcription start site (TSS) were abolished following treatment with the prototype BRD4 inhibitor, JQ1 (Fig. 1A). Other BET bromodomain proteins such as BRD2 were recruited to the *HOXB13* gene suggesting a certain degree of redundancy (Supplementary Fig. S1A). We recently reported the development of novel small molecule inhibitors (MA4-022-1, MA4-022-2, and SG3-179) that have the dual ability to inhibit the BET bromodomain proteins and a subset of kinases of the JAK family (23, 24). (Supplementary Fig. S2A). To validate *HOXB13* as a bona fide epigenetic target of BRD4, chromatin extracts prepared from the metastatic PC cell line, C4-2B were treated with Vehicle (DMSO), JQ1 (BET-inhibitor), MA4-022-2 (BET-kinase inhibitor) (23), the AR antagonists ENZ or Abiraterone acetate (ABR) (38). ChIP-qPCR was performed with anti-BRD4 or IgG control antibodies for BRAH1, BRAH2 and IGX1A (control) sites. BRD4 binds both BRAH1 and BRAH2 sites but not the control site, IGX1A, that was abolished in JQ1 and MA4-022-2 (MA4-2) treated cells (Fig. 1B–D). Consistently, BRD4 was recruited to the BRAH1/2 sites in another metastatic CRPC model cell line, 22Rv1, but not at the IGX1A control site (Fig. 1E–G). Further, analysis of ChIP-sequencing data revealed binding of BRD4 was reduced in DHT stimulated VCaP cells treated with ENZ and to a lesser extent with Bicalutamide (38) (Supplementary Fig. S1A).

To evaluate whether AR has a role in BRD4 recruitment at the *HOXB13* genomic locus, we first analyzed ChIP-sequencing data (GSE55064) and confirmed AR recruitment to the BRAH1/BRAH2 sites (Supplementary Fig. S1B). Next, we validated AR recruitment by ChIP-qPCR analysis in C4-2B cells and 22Rv1 cells (Fig. 1E, Supplementary Fig. S1C). Consistently, a decrease in BRD4 recruitment at the BRAH1/2 sites was diminished following AR blockade therapies, but not completely abolished in the CRPC model, C4-2B (Fig. 1B and C). In contrast, BRD4 was not recruited to the FOXA1 enhancer binding site previously reported to restrict HOXB13 expression to the prostatic lineage (39) (Supplementary Fig. S1D).

As acetylation of histone H3 and H4 at lysine residues are known to regulate the assembly of transcriptional co-activator complex at enhancer sites (40), we performed ChIP-qPCR with Histone H3K27, H4K5/K8/K12 and H4K16 acetylation specific antibodies to examine the epigenetic landscape at the *HOXB13* gene locus (Fig. 1E-I, Supplementary Fig. S1E-G). We observed a significant enrichment of the Histone H3K27 acetylation marks, characteristic of promoter/enhancers of transcriptionally active genes as well as H4K12ac marks at the BRAH1/BRAH2 enhancer but not at the IGX1A site (Fig. 1E-I). Intriguingly, the H4K12ac and to some extent H4K8ac but not the H4K5ac marks were enhanced in ENZ as well JQ1 treated CRPC cells at the BRAH1/BRAH2 sites, but not at the transcriptionally silent site (IGX1A) (Supplementary Fig. S1E-G). Further, the *HOXB13* enhancer region and TSS were enriched for RNA Pol II and Transcription Initiation Factor IID subunit 1 (TAF1) consistent with an actively expressed gene (Fig. 1E-F and I). These results suggest that the HOXB13 gene is in a transcriptionally permissive state despite anti-androgen treatment.

***HOXB13* gene expression is recalcitrant to anti-androgens but sensitive to Bromodomain-kinase inhibition**

To ascertain the molecular targets of the novel small molecule BET-kinase inhibitors in CRPCs we performed affinity proteomics (23, 24). Chemical affinity proteomic analysis confirmed BRD4 as one of the top binding proteins to the MA4-022-1 beads (Supplementary Fig. S2A). In addition, since the scaffold for the MA/SG compounds is based on the diaminopyrimidine scaffold, kinases such as NEK9 showed significant binding to the beads in the C4-2B cell extract (Supplementary Fig. S2A). However, RNA interference experiments revealed that knockdown of NEK9 had minimal impact on the growth of PC cells. In contrast to the short-half-life of JQ1, making it unsuitable for clinical purpose, the MA4-022-1 compound and analogs developed by our group are potent, highly soluble, and stable in aqueous media (23). To compare the sensitivity of CRPCs of the novel BET-kinase inhibitors versus Enzalutamide, we analyzed the anti-proliferative activity of the prototype BET inhibitor, JQ1, the three novel BET-kinase inhibitors, Enzalutamide and a control compound, MA3-068-1 (23). AR expressing CRPC cell lines (C4-2B, C4-2B ENZR (Enzalutamide resistant derivative of C4-2B) (2), 22RV1), the androgen responsive CRPC cell line VCaP, a normal prostate epithelial cell line, RWPE-1 as well as the AR negative cell lines DU145 and PC3 were evaluated (Supplementary Fig. S2C-F and Supplementary Fig. S3A-C). We observed that compared to Enzalutamide, the three BET-kinase inhibitors displayed potent anti-proliferative activity at sub-micromolar concentrations against C4-2B, C4-2B-ENZr, VCaP and metastatic CRPC cell line, 22Rv1 (Supplementary Fig. S2C-F).

Importantly, 22Rv1 that is highly resistant to the anti-androgen Enzalutamide (IC₅₀ 32 uM) retained high sensitivity to the novel BET-kinase inhibitors (IC₅₀ 1–2 uM) suggesting that these inhibitors can be used to overcome anti-androgen resistance (Supplementary Fig. S2D).

Subsequently, we analyzed HOXB13 mRNA levels in C4–2B cells depleted of BRD4 with specific siRNA or control siRNA (Fig. 2A). A specific decrease in HOXB13 mRNA levels was observed in BRD4 and HOXB13 knockdown cells but not in the control siRNA or AR siRNA transfected cells. However, HOXB13 depletion significantly decreased AR levels consistent with earlier reports (1). Importantly, an increase in AR and HOXB13 but not BRD4 was observed following treatment with the anti-androgen Enzalutamide (Fig. 2A). Decrease in HOXB13 had no effect on BRD4 expression in the absence of Enzalutamide with some effect in its presence (Fig. 2A).

To validate HOXB13 as a therapeutically relevant target of the novel BET-kinase inhibitor/s, we performed two independent RNA-sequencing analysis of C4–2B cells treated with MA4-022-1 and analogs (Supplementary Fig. S4A–B). A reduction in HOXB13 expression was observed in MA4-022-1 treated cells (Supplementary Fig. S4A) and in C4–2B cell treated with JQ1 or BET-kinase inhibitors in a repeat RNA sequencing analysis (Supplementary Fig. S4B).

Quantitative Reverse Transcriptase PCR (qRT-PCR) in CRPC lines revealed that HOXB13 mRNA expression was suppressed following treatment with JQ1, MA4-022-1 and analogs (Fig. 2B–D). In contrast, FOXA1 mRNA expression was not impacted by the BET inhibitors (Fig. 2B). As a control, we analyzed the expression levels of PSA and c-MYC and shown to be impacted by BET inhibition and observed them to be suppressed as reported (19) (Fig. 2C–2D; Supplementary Fig. S4C). Notably, HOXB13 expression was also suppressed in LNCaP cells grown in charcoal stripped media following treatment with BET inhibitors independent of DHT stimulation (Fig. 2E). Treatment of PC cells with GSK-126, an inhibitor of the catalytic subunit of the Polycomb repressive complex 2 (PRC2) did not decrease HOXB13 mRNA expression (Fig. 2E–F) or negatively impact C4–2B cell proliferation (Supplementary Fig. S5A–B). Thus, *HOXB13* gene activation is likely dependent on BRD4 mediated epigenetic regulation. To determine whether inhibition of BET bromodomain protein impacts the expression of other *HOXB* family members in *cis* or *trans*, we examined the expression of *HOX* genes *in cis* on Chromosome 17 as well as *HOXA13*, *HOXC13* and *HOXD13* located *in trans*. We did not detect the expression of *HOXB1* to *HOXB5* and *HOXB8* in VCaP cells. A marginal effect on *HOXB6* and *HOXC13* was observed. In contrast, *HOXA13* expression was significantly upregulated while *HOXB7* and *HOXD13* were downregulated following BET inhibition (Supplementary Fig. S4F). As HOXB13 was found to be expressed in the BT474 breast cancer cell line and associated with tamoxifen resistance (41), we examined the effect of BET-kinase inhibitors on HOXB13 mRNA levels and found it to be suppressed (Supplementary Fig. S4G). Collectively, our results indicate the epigenetic activation of *HOXB13* by bromodomain proteins may be a conserved regulatory mechanism in cancers of different tissue specificities.

Suppression of *HOXB13* expression induces apoptosis of CRPCs

To further confirm BRD4 mediated control of *HOXB13* protein expression, we silenced BRD2–4 and BRDT expression with selective silencing RNAs (siRNAs) (Fig. 3A). A significant reduction in *HOXB13* protein expression was observed when PC cells were transfected with BRD4 and BRDT siRNAs (Fig. 3A). Analysis of the kinetics of *HOXB13* expression revealed that the prototype BET inhibitor JQ1 as well as the novel BET-kinase inhibitors MA4-022-2 or SG3–179, caused a rapid reduction in *HOXB13* protein expression (15h in MA4-022-2 treated cells versus 48h in JQ1 treated VCaP cells) (Fig. 3B). Moreover, a rapid induction of apoptosis was observed as seen by c-PARP cleavage that was concomitant with *HOXB13* downregulation (Fig. 3B-third panel) and to a much lesser extent in ENZ (Fig. 3C). c-MYC was observed to be downregulated with JQ1 as well as by MA4-022-2 and SG3–179, consistent with the earlier reports (Fig. 3B). Immunoblotting also revealed a significant reduction in *HOXB13* protein expression with various BET inhibitors in VCaP, C4–2B and E8 (a mouse PC model), but was not impacted in cells treated with ENZ (Fig. 3C–E).

To determine whether BRD4 loss phenocopies the effect of *HOXB13* depletion we performed siRNA mediated knockdown of BRD4 or *HOXB13* or NEK9. Loss of BRD4 or *HOXB13* but not NEK9 or control siRNA led to a significant decrease in cell proliferation (Fig. 3F). We performed a rescue experiment with exogenously expressed wild-type *HOXB13* that is not under the epigenetic control of BRD4. *HOXB13*-WT could significantly rescue the cytotoxicity induced by the treatment of C4–2B cells with JQ1 at 0.5 μ M concentration ($p < 0.001$) (Fig. 3H). A complete lack of rescue could be attributed to the low transfection efficiency characteristic of PC cells. Moreover, we observed that migration of 22Rv1 was significantly impacted following treatment with MA4-022-2 and to a lesser extent with JQ1 but not ENZ or ABR (Fig. 3H–I). Collectively, these results suggest that *HOXB13* is a critical transcriptional target of BRD4 in CRPCs.

HOXB13 is critical for castration resistant prostate cancer growth

To delineate whether *HOXB13* function is indeed essential in CRPC progression and promoting anti-androgen resistance, we generated isogenic WT (parental) and *HOXB13* pKO cell lines (partial knockout of *HOXB13*) in the C4–2B genetic background using the CRISPR/Cas9 gene editing technology, as complete deletion was lethal (Fig. 4A). We observed that haplo-insufficiency of *HOXB13* altered the sensitivity of C4–2B cells to BET inhibitors by 2-fold and ENZ sensitivity by 3-fold (Supplementary Fig. S5A). To further examine whether reduced *HOXB13* expression mitigates CRPC xenograft tumor growth *in vivo*, we injected the isogenic C4–2B and C4–2B *HOXB13*pKO cells subcutaneously in intact and castrated male SCID mice. Compared to the isogenic parental cells, the *HOXB13*pKO C4–2B cells were significantly impaired in their ability to form tumors in intact and particularly in castrated male mice (Fig. 4B–C). In contrast to other *HOXB13* paralogs, *HOXD13* levels were significantly increased following the reduction in *HOXB13* expression in residual *HOXB13* pKO tumors harvested from castrated mice (Supplementary Fig. S5B). In addition, we also observed an increase in AR mRNA and c-MYC, levels, that was exacerbated following castration (Fig. 4D). In addition to C4–2B model, we also assessed the effect of genetic ablation of *HOXB13* in VCaP cells. Genetic reduction of

HOXB13 abrogated VCaP xenograft tumor growth suggesting an extreme transcription factor dependency (Fig. 4E–F). This result demonstrates that not only does HOXB13 collaborate with androgen dependent AR signaling to establish tumors in intact male mice, but can also compensate for the lack of androgen to promote CRPC growth.

To determine whether the BET inhibitors phenocopy the genetic ablation of HOXB13 to impact growth of CRPC tumors, castrated male immunocompromised mice were implanted subcutaneously with C4–2B or intact mice with VCaP cells which were castrated following tumor initiation (Fig. 4G and Supplementary Fig. S5C). Mice were treated with vehicle or the experimental compound, for 5 days a week for 2 weeks (C4–2B CRPC xenograft tumors) or 4 weeks (VCaP CRPC xenograft tumors) at 50 mg/kg body weight. A significant inhibition of C4–2B growth was observed with JQ1 and/or MA4-022-2 (Fig. 4G–H) and to a lesser extent in VCaP (Supplementary Fig. S5C). Histological examination of the liver, kidney, and brain did not reveal any gross morphological abnormalities and the weights remained constant (Supplementary Fig. S5D–E), suggesting that novel compound, MA4-022-2, or its derivatives could be potential therapeutics for CRPCs for pre-clinical development.

To evaluate the therapeutic potential of BET inhibitors in preventing the dissemination of HOXB13 positive PCs from the primary site, we analyzed for the presence of circulating tumor cells (CTCs). To obtain prostate-specific CTCs we collected blood from castrated male SCID mice harboring VCaP xenograft tumors that were either treated with Vehicle (DMSO) or JQ1 (n=5 in each group) for 4 weeks. In contrast to the control (DMSO) group, there was a significant drop in the number of CTCs, as well as HOXB13 positive CTCs with the BET inhibitor treatment (677 in DMSO group versus 63 in JQ1 group) (Supplementary Fig. S5F).

BET-kinase inhibitor-responsive HOXB13 target gene set (HOTBIN10) comprise a proliferative associated transcriptional network

We performed integrative bioinformatics analysis to identify those targets that are potentially regulated by HOXB13. As a first step to filter non-specific candidate genes, we isolated Differentially Expressed Genes (DEGs) that were commonly affected by the three novel closely related BET-kinase inhibitors. Integration of RNA-sequencing data sets for the three analogs revealed 195 genes (Fig. 5A). Subsequently, we performed a second round of integrative analysis by comparing the 195 DEGs against the DEGs isolated from the HOXB13pKO/parental C4–2B (DMSO) (2012) list of DEGs, using a stringent padj values cutoff = 0.001 to identify HOXB13 specific targets that are responsive to the BET inhibitors (Fig. 5B). This analysis revealed 124 differentially expressed HOXB13 target genes that were also significantly impacted by BET inhibition (Fig. 5B–C). Gene enrichment analysis of these HOXB13 target genes (HOXTAR124) revealed an overrepresentation of genes involved in chromatin structure and organization, cell cycle progression, DNA replication and repair pathways (Fig. 5D). Consistently, cell cycle analysis revealed that the BET-kinase inhibitor caused a G1/S inhibition similar to JQ1 at low concentrations and arrested cells at G2/M at higher concentrations in all three CRPC models (Fig. 5E–G). In contrast, ENZ had no impact on cell cycle progression of CRPCs and was similar to the vehicle control.

HOXB13 target genes upregulated in treatment resistant metastatic CRPCs

Compound mutant mice (*NKX3.1^{CreERT2/+}/PTEN^{flox}/p53^{flox}*; Np53) with luminal prostate epithelial specific genetic deletion of *PTEN* and p53 tumor suppressor develop CRPC that harbors molecular features of metastatic human CRPCs (42). To determine whether HOXB13 regulated pro-proliferative function is conserved in multiple organisms we analyzed HOXB13 target gene expression in public gene expression dataset of mCRPC tumors derived from the Np53 mice (GSE92721; Supplementary table S4). Gene Set Enrichment Analysis (GSEA) revealed a positive enrichment for ~109 of the 124 HOXB13 target genes (HOXTAR109) originally identified (Fig. 6A–C); specifically, in tumors derived from Np53 compared to *NKX3.1^{CreERT2/+}* mice (N) (NES=1.644, p=0.0) (Fig. 6B) or in mice with *PTEN* deletion (NP) (NES=2.064, p=0.0) (Fig. 6C). In contrast, a significant enrichment was not observed in NP vs N mice (NES=1.215, p=0.156) (Fig. 6A). Notably, analysis of HOXB13 target gene expression from Np53CRPC mice treated with Abiraterone, a subset referred to as exceptional non-responders due to their propensity to develop accelerated tumor phenotypes and metastasis, revealed a significant enrichment (NES= 2.115, p=0.0) suggesting that activation of the HOXB13 pathway correlates with severity of disease progression (Supplementary Fig. S5G, Supplementary table S5).

To further ascertain the clinical significance of HOXTAR124, we analyzed their expression in microarray data obtained from de-identified 194 primary and 29 metastatic tumors from the Moffitt Cancer Center Tissue Core (TCC) Data Set (Fig. 6D–F; Supplementary tables S6–S8). Heat maps were generated for 124 unique genes (Fig. 6D) which revealed that a 10-gene core set (HOXB13 target genes responsive to BET inhibitors) HOTBIN10, could effectively distinguish primary from metastatic prostate tumors by first component of Principle Component Analysis (PCA) at 86.3% (Fig. 6E). Violin plots also revealed a clear stratification of HOTBIN10 in primary versus metastatic tumors with lower expression segregating with primary and higher expression with metastatic PCs (Fig. 6F). The HOTBIN10 gene sets were independently validated for their ability to separate primary from metastatic tumors by first component of PCA at 70.4% in the GSE21034 data set (36) (Fig. 6G–I; Supplementary table S9).

To confirm the association of HOTBIN10 with PC progression we analyzed expression in the different mouse cohorts that represent different stages of progression. The subset of the Np53CRPCs treated with abiraterone and described as exceptional non-responders due to their distinct pathological features showed an elevated HOTBIN10 gene expression (Supplementary Fig. S5H). Combined, these results indicate HOTBIN10 genes as a hallmark of lethal metastatic CRPCs.

BRD4-HOXB13 nexus co-opts a conserved pro-proliferative transcriptional network that is insensitive to AR blockade

To determine whether HOTBIN10 gene expression can be modulated by genetic or pharmacological ablation of HOXB13, we silenced HOXB13 expression by siRNA in CRPCs (C4–2B or VCaP) or treated cells with the BET-kinase inhibitor MA4-022-2 and found a corresponding decrease in the expression of HOTBIN10 genes (Fig. 7A–B). Importantly, HOTBIN10 gene expression remained significantly high in C4–2B despite ENZ

treatment compared to LNCaP (Fig. 7C). Importantly, c-MYC was not impacted by the loss of HOXB13 (Fig. 7A). Search Tool for the Retrieval of Interacting Genes (STRING) analysis revealed that the genes comprising HOTBIN10 may form an evolutionarily conserved protein-protein interaction network (Fig. 7D). Ablation of BRD4, HOXB13 and AR using siRNAs revealed that HOTBIN10 gene expression was not affected following Control and AR silencing in DMSO (vehicle) or ENZ treated cells (Fig. 7E–F). Consistently knockdown of BRD4 or HOXB13 suppressed expression of the HOTBIN10 genes (Fig. 7E–F).

Given the association of HOTBIN10 gene expression with AR independence, we built gene expression prediction models to stratify patients into those with primary versus metastatic PC. Each prediction model was trained on the basis of 150 patients in the MSKCC validation dataset and came out with a threshold score (AUC 0.711) (Supplementary Fig. S6A). The optimal models and corresponding thresholds were then tested on Moffitt TCC data. Analysis of the HOTBIN10 gene set revealed an overall accuracy of 95.4% and AUC of 0.99 in classifying primary and metastatic prostate cancer in the Moffitt TCC data set (Supplementary Fig. S6B). A recent study of 27 cases of mCRPC patients treated with first-line abiraterone, revealed that a subset of patients (6/6) with HOXB13 positive expression in circulating tumor cells (CTCs) died within a year (18). To further examine the significance of HOTBIN10 in CRPC dissemination, we examined the expression of HOTBIN10 in single cell RNA-sequencing data from these patients (GSE67980) (Supplementary table S10). We observed significant clonal heterogeneity in the single CTCs based on their HOXB13 and HOTBIN10 gene expression profiles (Supplementary Fig. S6C). Of these, the expression of two genes, *AURKB* and *MELK*, correlated with *HOXB13* suggesting that AURKB inhibitors could be used to target high-risk HOXB13 positive metastatic PCs (Supplementary Fig. S6D–E). We therefore tested the activity of AURKB inhibitor Barasertib currently in Phase I/II clinical trials for lymphoid and leukemia. While Barasertib showed significant anti-proliferative activity in CRPC models, the BET-kinase inhibitors performed better in comparison (Supplementary Fig. S7A–C). We observed that VCaP cells treated with Enzalutamide had increased expression of AURKB, suggesting a novel mechanism of resistance (Supplementary Fig. S7D). Combined, our studies not only uncovered a role for the BRD4-HOXB13-AURKB axis in anti-androgen resistance of CRPCs but small molecule inhibitors to target this regulatory circuit (Supplementary Fig. S7E).

Discussion

Prostate Cancer (PC) remains a leading cause of cancer related deaths among men worldwide. The prognosis for patients diagnosed with metastatic Castration Resistant Prostate Cancer (CRPC) is bleak as the disease ceases to respond to the current first and second generation anti-androgen therapies, indicating that the cancer cells rewire their transcription programs to survive in the absence of their fuel, androgen. Importantly, the molecular mechanisms by which CRPCs evade androgen deprivation therapies (ADT) are varied, suggesting that identifying and targeting AR dependent and independent pathways are critical to improve patient outcomes. In this report, we demonstrate that HOXB13 gene expression is epigenetically regulated by the BET family of bromodomain proteins that is

largely androgen-independent. BRD4-HOXB13 nexus in turn promotes an androgen-independent transcriptional network of genes implicated in cell cycle, DNA damage response, nucleotide metabolism and metastasis. Thus, a nexus of transcription factor (HOXB13) and an epigenetic regulator (BRD4) encoded by two developmental genes that are re-expressed in CRPCs weans cells of androgen requirement and drives new dependency.

In silico analysis revealed that in addition to BRD4, peaks for BRD2 and BRD3 were detected at the *HOXB13* enhancer region in VCaP cells suggesting potential recruitment (Supplementary Fig. S1A). Moreover, we found that silencing of individual BRD members with gene specific siRNAs (BRD 2, 3 or 4) reduced *HOXB13* expression to a significant extent but not completely indicating potential redundancy in functionality. Similarly, BRD2/3/ and 4 have been identified as either regulators of AR target gene expression or of AR splicing in CRPCs underscoring the importance of targeting them to overcome anti-androgen resistance (19, 20). Importantly, the novel bromodomain-kinase inhibitors due to their pan-BRD inhibitory activity should be very useful to inhibit multiple BRDs that may compensate for each other to thwart the development of resistance.

The molecular role of HOXB13 in PC has been uncertain. HOXB13 was initially reported to prevent transactivation of hormone regulated AR target genes, through its interaction and sequestration of AR from binding its cognate responsive elements (8, 9). Subsequently Kim *et. al.*, have also reported that HOXB13 is overexpressed in hormone refractory tumors and promotes androgen-independent growth of LNCaP cells by regulating the RB-E2F signaling to inhibit the tumor suppressor p21Waf and promote proliferation (11). In addition to full length AR, HOXB13 has been shown to interact with the AR-V7, a splice variant that lacks the ligand binding domain and promotes the establishment of AR cistrome in CRPCs (12). The function of HOXB13 in PC may also be regulated via its interaction with the MEIS proteins. Lower expression of these potential tumor suppressors MEIS1 and 2 is associated increased likelihood of metastasis (43). We observed that CRPCs deploy HOXB13 to upregulate a pro-proliferative BRD4-HOXB13 dependent transcriptional network in response to ADT. Key members of the pro-proliferative network that we identified in this study include a core HOXB13 effector 10 gene set (HOTBIN10-HOXB13 target genes response to BET inhibitors) that can remarkably stratify human prostate tumors into the normal, primary and metastatic groups and has been validated in independent datasets (~400 biopsies). In addition, our study reveals that the HOXB13 transcriptional network was insensitive to AR depletion or blockade but responsive to the inhibition of BRD4-HOXB13 epigenetic axis. Importantly, identification of BRD4 as an epigenetic regulator of *HOXB13* even under conditions of androgen deprivation uncovers a novel mode of targeting and inhibiting *HOXB13* expression in PCs. Our studies therefore unravel for the first time not only an epigenetic mechanism underlying *HOXB13* gene regulation in PC, but also the therapeutics to target HOXB13 pathway, a promoter of CRPC growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. BRD4 is recruited to the *HOXB13* gene locus in prostate cancer

A, Schematic of *HOXB13* genomic region on Chromosome 17 with the BRD4 binding peaks in VCaP cells (GSE55064). BRD4 binding peaks BRAH1 and BRAH2 upstream of the *HOXB13* TSS. **B-D**, ChIP-qPCR with anti-BRD4 or anti-IgG in C4-2B cells prepared from vehicle (DMSO), JQ1 (2.5 μ M), MA4-022-2 (2.5 μ M), and ENZ (5 μ M) treatments for 16h for BRAH1, BRAH2 and IGX1A (control) sites (Data are mean $\pm$ SEM of two independent experiments. **** $p < 0.0001$; ** $p < 0.005$; * $p < 0.05$; ns-not significant, ANOVA. **E-G**, ChIP-qPCR for H3K4me1, H3K27ac, H4K12ac, RNA Pol II, BRD4, TAF1, and AR at BRAH1 and BRAH2, IGX1A in 22Rv1 cells (Data are mean $\pm$ SEM; n=3

replicates). **H-I**, ChIP with RNA Pol II and various histone modification specific antibodies in C4-2B cells followed by qPCR analysis for BRAH1, BRAH2, IGX1A (control) and HOXB13 Transcription Start Site (TSS) in C4-2B cells (Data are mean $\pm$ SEM; n=3 replicates).

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Fig. 2. HOXB13 is a transcriptional target of BRD4

A, C4-2B cells transfected with control, HOXB13 or AR siRNA or BRD4 siRNA in the presence or absence of 5 uM Enzalutamide. HOXB13, AR and BRD4 mRNA levels were determined by RT-qPCR. Actin was used as the normalization control (Data are mean $\pm$ SEM; n=3; replicates) **** p<0.0001; ** p<0.005; * p<0.01; ns-not significant, ANOVA. **B**, VCaP cells grown in charcoal stripped media for 2 days were treated with 5 uM ENZ or 5 uM ABR or 2.5 uM JQ1 or 2.5 uM MA4-022-2 for 16h. qRT-PCR was performed for FOXA1, HOXB13 and Actin (n=3; replicates). **** p<0.0001; * p<0.05; ns-not significant, ANOVA. **C**, C4-2B cells treated with MA4-022-1, MA4-022-2 or JQ1 at indicated

concentrations and the expression of HOXB13, PSA and Actin was determined by qRT-PCR. (n=3; replicates) **** $p<0.0001$; ** $p<0.005$; * $p<0.05$; ANOVA. **D**, C4-2B cells were treated with vehicle (DMSO) or with 2.5uM of JQ1 or MA4-022-2 or SG3-179 or ENZ and the expression of HOXB13, c-MYC and Actin were determined by qRT-PCR. Actin was used as the normalization control (n=3; replicates). **** $p<0.0001$; * $p<0.01$; ns-not significant, ANOVA. **E**, LNCaP cells grown in charcoal stripped media were treated with 2.5 uM of vehicle (DMSO), JQ1, MA4-022-2, ENZ or GSK-126 for 16h followed by treatment with 100 nM of DHT for 6h. Expression of HOXB13 and Actin was determined by qRT-PCR (n=3; replicates) **** $p<0.0001$; ** $p<0.005$; * $p<0.05$; ns-not significant, ANOVA. **F**, VCaP cells were treated with 2.5 uM of vehicle (DMSO), Control (MA3-068-1), MA4-022-1, ENZ, GSK-126, Fedratinib, iBET-762, SG3-179 or JQ1. Expression of HOXB13 and Actin was determined by qRT-PCR. Data is representative of 2 independent experiments. **** $p<0.0001$; ** $p<0.005$; ns-not significant, ANOVA.

Fig. 3. Rapid turnover of HOXB13 protein in CRPCs treated with BET-kinase inhibitors

A, Immunoblot analysis of HOXB13 expression following transfection of C4-2B cells with various BRD silencing RNAs or HOXB13 siRNA. Actin is used as a loading control. **B**, Immunoblot analysis for HOXB13, c-MYC, cPARP, and Vinculin in VCaP cells treated with vehicle (DMSO), 2.5 uM of JQ1 or MA4-022-2 or SG3-179. **C**, Immunoblot analysis of HOXB13, c-Myc and cPARP expression following treatment with vehicle (DMSO), 2.5 uM of JQ1 or MA4-022-1 or MA4-022-2 or SG3-179 or ENZ for 16h in VCaP cells. **D**, The mouse PC cell line E8 was treated with vehicle (DMSO), 2.5 uM of JQ1 or MA4-022-2 or SG3-179 or ENZ for 16h and the whole cell lysate was immunoblotted for HOXB13 and

Actin. **E**, Immunoblot to analyze HOXB13 expression in C4-2B cells treated with 2.5 μ M of JQ1 or MA4-022-2 or SG3-179. Actin used as a loading control. **F**, LNCaP cells transfected with control, BRD4, HOXB13 and NEK9 siRNAs. Cells viability was determined by Trypan blue assay. Data are represented as mean $\pm$ SEM. **** $p < 0.0001$, ns= not significant, ANOVA. **G**, C4-2B cells transfected with vector or HOXB13-WT constructs were treated with 0.5 μ M JQ1 and cell viability was measured by CellTitre-Glo analysis (Y-axis= RLUs; n=9 replicates; $p < 0.0001$, ANOVA. **H-I**, 22Rv1 cells were treated with vehicle, 1 μ M of JQ1, or MA4-022-2 or ENZ or ABR and transwell cell migration assay was performed. For each insert, images were captured at 100x total magnification. The number of cells in each field is reported as an average of 5 picture fields. Data are mean $\pm$ SEM. * $p < 0.01$ *** $p < 0.0001$, ns-not significant, ANOVA.

Fig. 4. BET bromodomain kinase inhibitors suppress CRPC xenograft tumor growth

A, Whole cell extracts of isogenic pairs of C4-2B parental and HOXB13pKO cell lines (1 and 2) were immunoblotted for HOXB13 and Actin. **B-C**, Intact and castrated male SCID mice were injected subcutaneously with parental C4-2B or HOXB13pKO cells. Tumors were harvested at the end of 7 weeks and tumor weights were taken * $p < 0.05$, ** $p < 0.005$, *** $p = 0.0001$, ANOVA (Sidak's multiple Comparison test). **D**, qRT-PCR analysis for HOXB13, NKX3.1, PSA, cMYC, AR and Actin in tumors isolated from isogenic C4-2B WT and HOXB13pKO under intact and castrated conditions. $n = 3$ mice; 3 replicates each (total = 9). ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$, ns, not significant, ANOVA (Sidak's

multiple Comparison test). **E-F**, Intact male SCID mice were injected subcutaneously with VCaP cells following parental or HOXB13KO partial ablation. Tumor volumes were measured by calipers. (n=12/cohort); *** $p < 0.0001$. Heterogeneous variance was observed between the VCaP-parental and HOXB13pKO groups (Levene F test $p < 0.01$). Two-sided two-sample t-test under unequal variance was therefore performed. **G-H**, castrated male SCID mice were injected subcutaneously with C4-2B cells. When tumors reached 100 mm³ to 150 mm³ the mice were injected with vehicle, JQ1 or MA4-022-2 (50 mg/kg of body) for 5 days a week for 2 weeks ($n=10-12$ mice/group). Tumor volumes were measured by calipers. Assumptions about homogeneous variance and normality were satisfied (Bartlett's test $p=0.08$ and Shapiro normality test $p=0.11$). ** $p=0.0005$ and * $p=0.0055$. One-way ANOVA test and Sidak's multiple comparison.

Fig. 5. Identification of HOXB13 transcriptome responsive to BET inhibitors

A, Integrative analysis for Differentially Expressed Genes (DEGS) that are common and significantly affected by the three related BET-kinase inhibitors- MA4-022-1, MA4-022-2 and SG3-179 compared to vehicle (DMSO). **B**, Integrative analysis reveals 124 genes commonly affected by BET bromodomain inhibition and HOXB13 genetic deletion. **C**, List of 124 genes modulated by BET inhibition or HOXB13 genetic reduction with hierarchical clustering for genes. Expression values were standardized to mean = 0 and standard deviation = 1 and hierarchically clustered. White color corresponds to FC = 0. **D**, Gene ontology analysis reveals biological pathways impacted following BET bromodomain

inhibition or HOXB13 genetic reduction. **E-G**, Cell cycle analysis of CRPC cell lines; **E**, C4-2B, **F**, 22Rv1 and **G**, C4-2B ENZR treated with DMSO (vehicle) 0.5 uM, 1 uM and 2.5 uM of JQ1, MA4-022-1, MA4-022-2, SG3-179 or ENZ.

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Fig. 6. HOTBIN10 genes can stratify primary from metastatic human prostate cancers
A-D, Gene set Enrichment Analysis (GSEA) from the public gene data set (GSE92721) for HOXB13 target genes (124). GSEA compares expression of the 109 conserved HOXB13 targets in 4 cohorts; **A**, NP (intact and castrated) vs control N prostate (N); **B**, NPp53 (intact and castrated) vs. control mouse prostate (N), **C**, NPp53 (intact and castrated) vs. NP, **D**, Heat map of HOTBIN10 genes in Moffitt TCC data set. Red bars indicate metastasis. **E**, PCA of HOTBIN10 gene set in Moffitt TCC Data Set (total n=223 =194 primary and 29 metastatic tumors). **F**, Violin plots of HOTBIN10 genes in primary versus metastatic PCs in Moffitt TCC Data Set. **G**, Heat map of HOTBIN10 genes in MSKCC (Taylor) data set

(GSE21034). Red bars indicate metastasis. **H**, PCA analysis modeling HOTBIN10 in 179 samples (29 adjacent normal, 131 prostate adenocarcinomas and 19 metastatic tumors) in GSE21034. **I**, Violin plots of HOTBIN10 genes in primary versus metastatic PCs in GSE21034.

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Fig. 7. HOTBIN10 gene expression is insensitive to AR blockade in CRPCs

A, qRT-PCR analysis of HOTBIN10 genes, HOXB13 and c-MYC expression in C4-2B cells transfected with control or HOXB13 siRNAs. Actin is used as a normalization control. Data are average of two independent biological replicates. **B**, Treatment of VCaP cells with vehicle (DMSO) or 2.5 uM MA4-022-2 followed by qRT-PCR for HOTBIN10 and Actin, n=3 replicates/condition. **C**, qRT-PCR analysis of HOTBIN10 gene expression in C4-2B and LNCaP cells following treatment with vehicle (DMSO) or 5 uM ENZ for 16h. **D**, STRING analysis of HOTBIN10 as an interconnected network and regulated by BRD4. PPI enrichment p-value < 1.0e-16. Dark lines for HOXB13 and BRD4 represent the new

connection identified in this study. **E-F**, C4-2B transfected with Control, HOXB13, BRD4 or AR siRNAs. 48h later cells were treated with vehicle (DMSO) or 5uM ENZ for 16h and analyzed for HOTBIN10 gene expression by qRT-PCR (Data are mean $\pm$ SEM; n=3 replicates).

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