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Supplemental Information

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Supplementary Information

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Jung Kim[1,#], Yongik Lee[1,#], Xiaodong Lu[1], Bing Song[1], Ka-Wing Fong[1], Qi Cao[3], Jonathan Licht[1,2,4], Jonathan C. Zhao[1,2,*], Jindan Yu[1,2,5,*]

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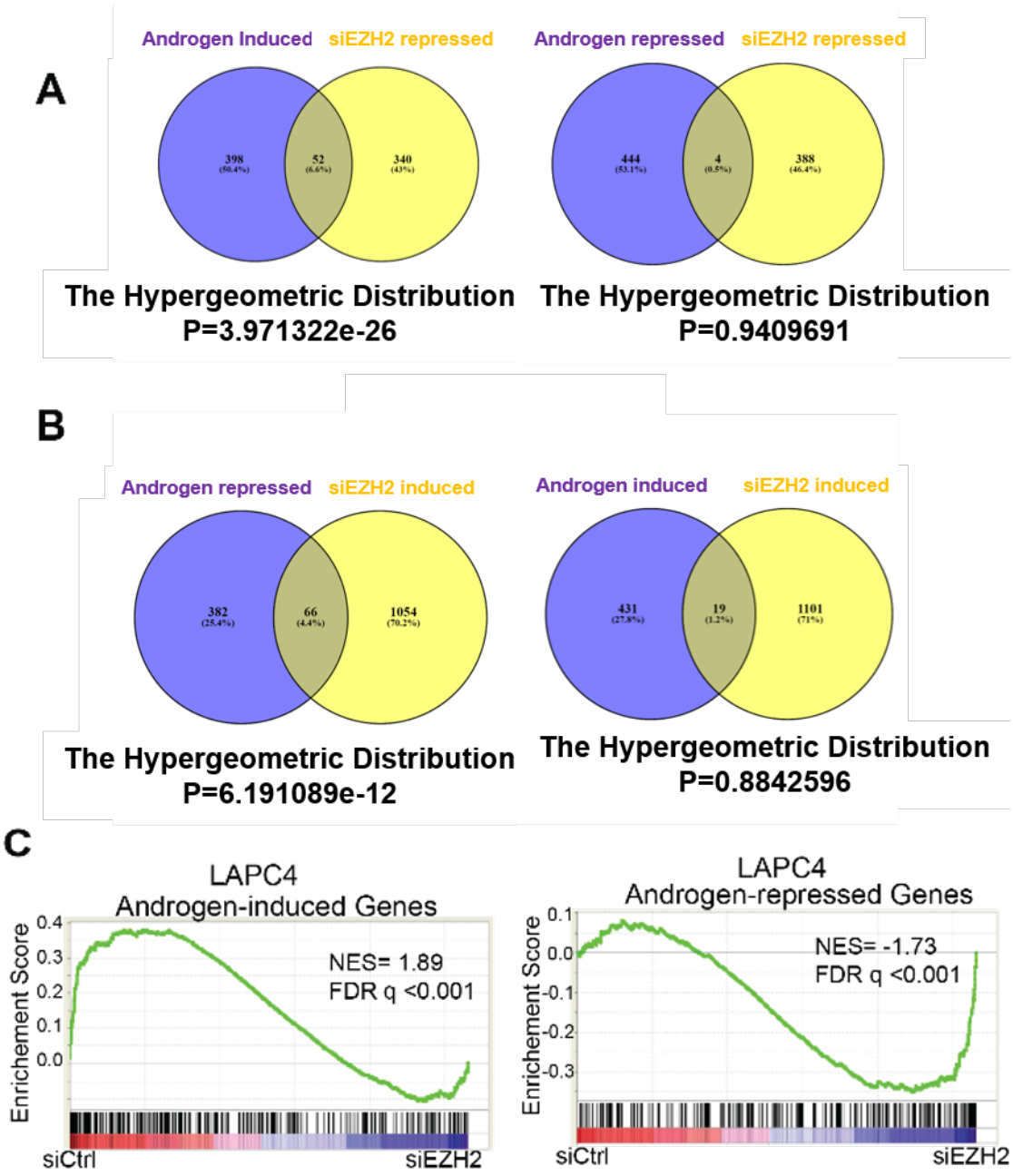

Figure S1. EZH2 positively regulates AR signaling, related to Figure 1. Venn diagram showing overlap between genes that were activated (**A**) or repressed (**B**) by EZH2 with those that are induced or repressed by androgen in LNCaP cells. (**C**) R1881 induced genes are enriched for downregulation upon EZH2 knockdown (FDR q <0.001), whereas R1881 repressed genes are enriched for restoration upon EZH2 knockdown (FDR q=0.003). GSEA was utilized to examine the expression of R1881-induced and -repressed gene sets in LAPC4 cells with control (siCtrl) or EZH2 knockdown (siEZH2), which were profiled by microarrays.

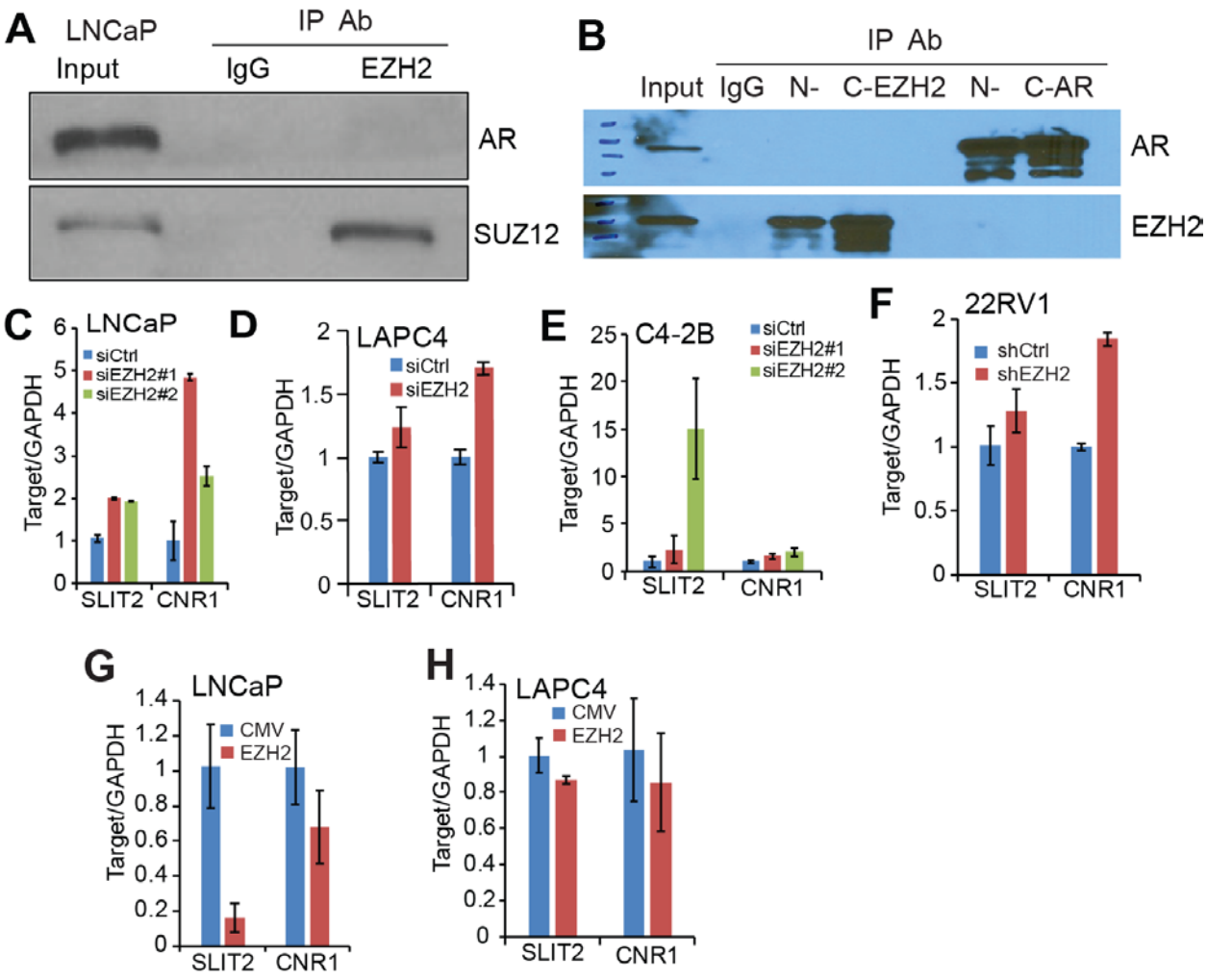

Figure S2. EZH2 and AR does not interact in LNCaP cells, related to Figure 2. (**A**) LNCaP cell lysate was pulled down with IgG or EZH2 antibody followed by immunobloting with SUZ12, a positive control, and AR antibodies. (**B**) LNCaP total cell lysate was pulled with either N or C-terminal epitope EZH2 or AR antibodies followed by immunobloting with AR and EZH2 antibodies. (**C-D**) QRT-PCR showing that EZH2 knockdown increases its well-known epigenetic targets, SLIT2 and CNR1, in LNCaP and LAPC4 cells. (**E-F**) QRT-PCR showing that EZH2 knockdown increased SLIT2 and CNR1 expression in C4-2B and 22Rv1. (**G-H**) EZH2 overexpression decreased SLIT2 and CNR1 in LNCaP and LAPC4 cells.

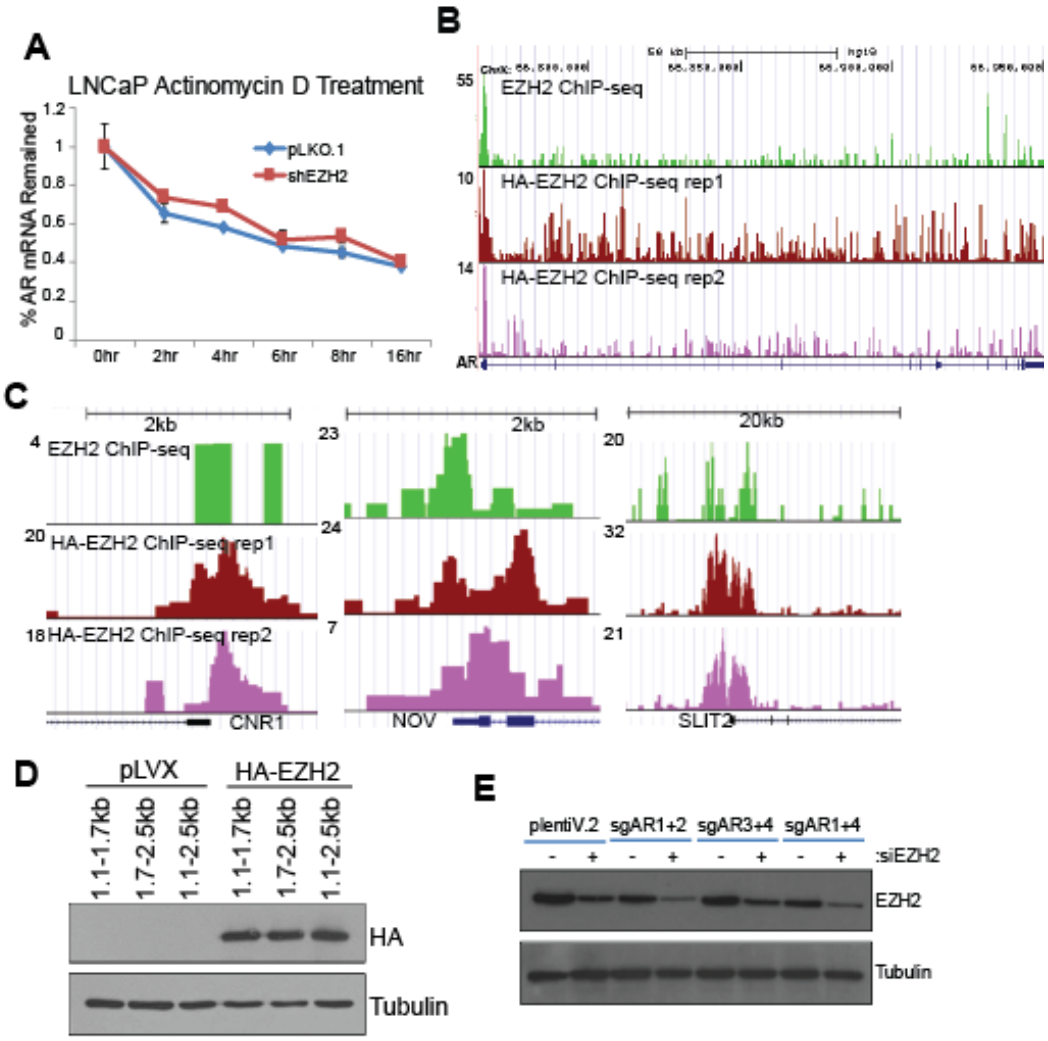

Figure S3. EZH2 directly induces AR gene transcription, related to Figure 3.
(A) EZH2 knockdown does not reduce AR mRNA stability. LNCaP pLKO.1 and EZH2 knockdown cells were plated and treated with actinomycin D for 2, 4, 6, 8, and 16 hrs prior to RNA isolation followed by qRT-PCR.
(B-C) EZH2 ChIP-seq was performed in LNCaP cells with antibody targeting endogenous EZH2 (top panel). HA-EZH2 ChIP-seq were performed using an anti-HA antibody in LNCaP cells with ectopic HA-EZH2 overexpression. Two biological replicates are shown (middle and bottom panel). EZH2 and HA-EZH2 *ChIP-seq* genome browser tracks for the entire AR gene (**B**) in LNCaP. Shown in (**C**) are EZH2 occupancy at the promoters of CNR1, NOV, and SLIT2 genes, which are previously reported targets of EZH2.
(**D**) HA-EZH2 overexpression confirmed by western blot analysis. 293T cells were transfected with either pLVX or HA-EZH2 along with different AR promoter constructs: 1.1-1.7kb, 1.7-2.5kb, or 1.1-2.5kb.
(**E**) EZH2 knockdown confirmed by western blot analysis. LNCaP cells were infected with lentiCRISPR/Cas9 containing pLENTI.V2 control, sgAR1+2, sgAR3+4, or sgAR1+4 for 48 hrs followed by EZH2 knockdown by transfection. EZH2 knockdown was validated using EZH2 immunoblot with tubulin loading controls.

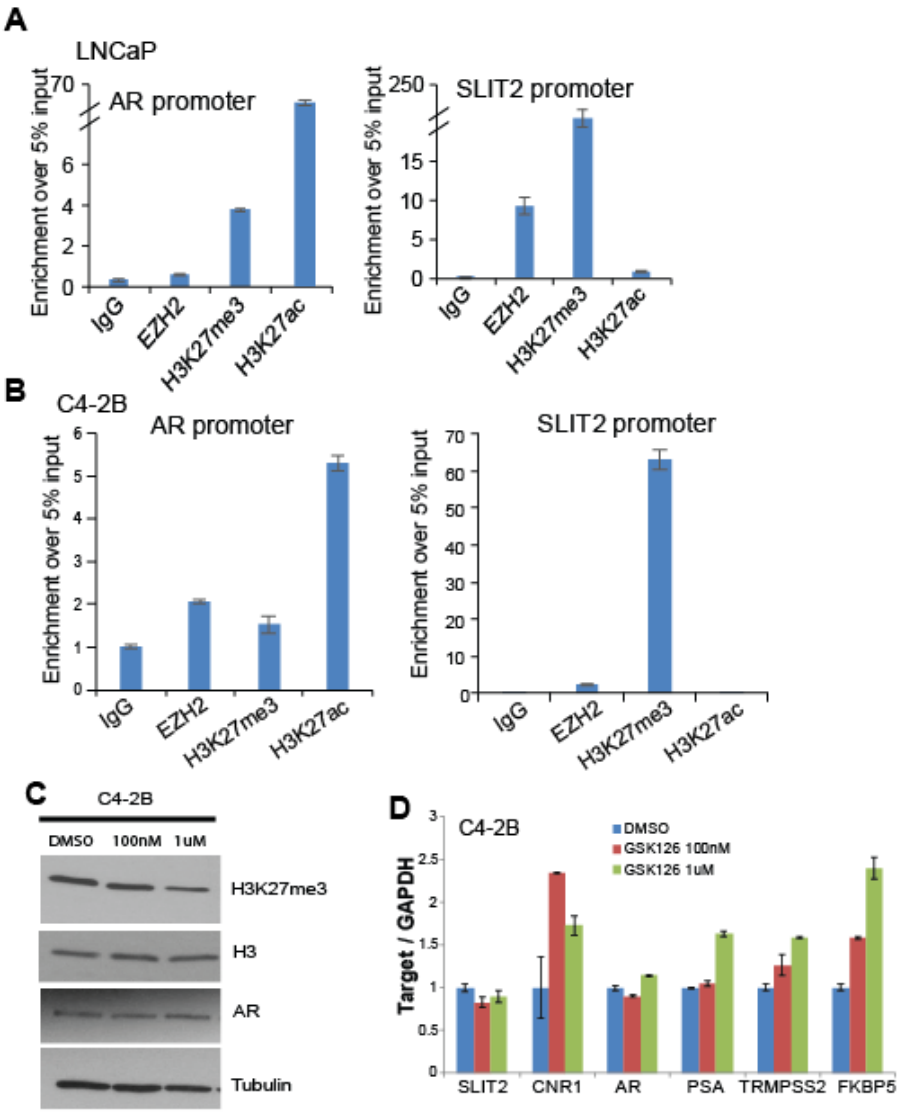

Figure S4. EZH2 activates AR through methylation-independent mechanisms, related to Figure 4.

(A-B) EZH2, H3K27me3, H3K27ac, and IgG ChIP was performed in LNCaP **(A)** and C4-2B **(B)** cells and then analyzed by qPCR for enrichment at the AR and SLIT2 promoters. Data shown are mean (±SEM) of technical replicates from one representative experiment of two.
(**C-D**) EZH2 methyltransferase inhibitors did not alter AR expression in C4-2B cells. C4-2B cells were treated with indicate doses of GSK126 for 72hrs and the cell lysates were subjected to **(C)** western blot analysis and **(D)** qRT-PCR. Data shown are mean (±SEM) of technical replicates from one representative experiment of three.

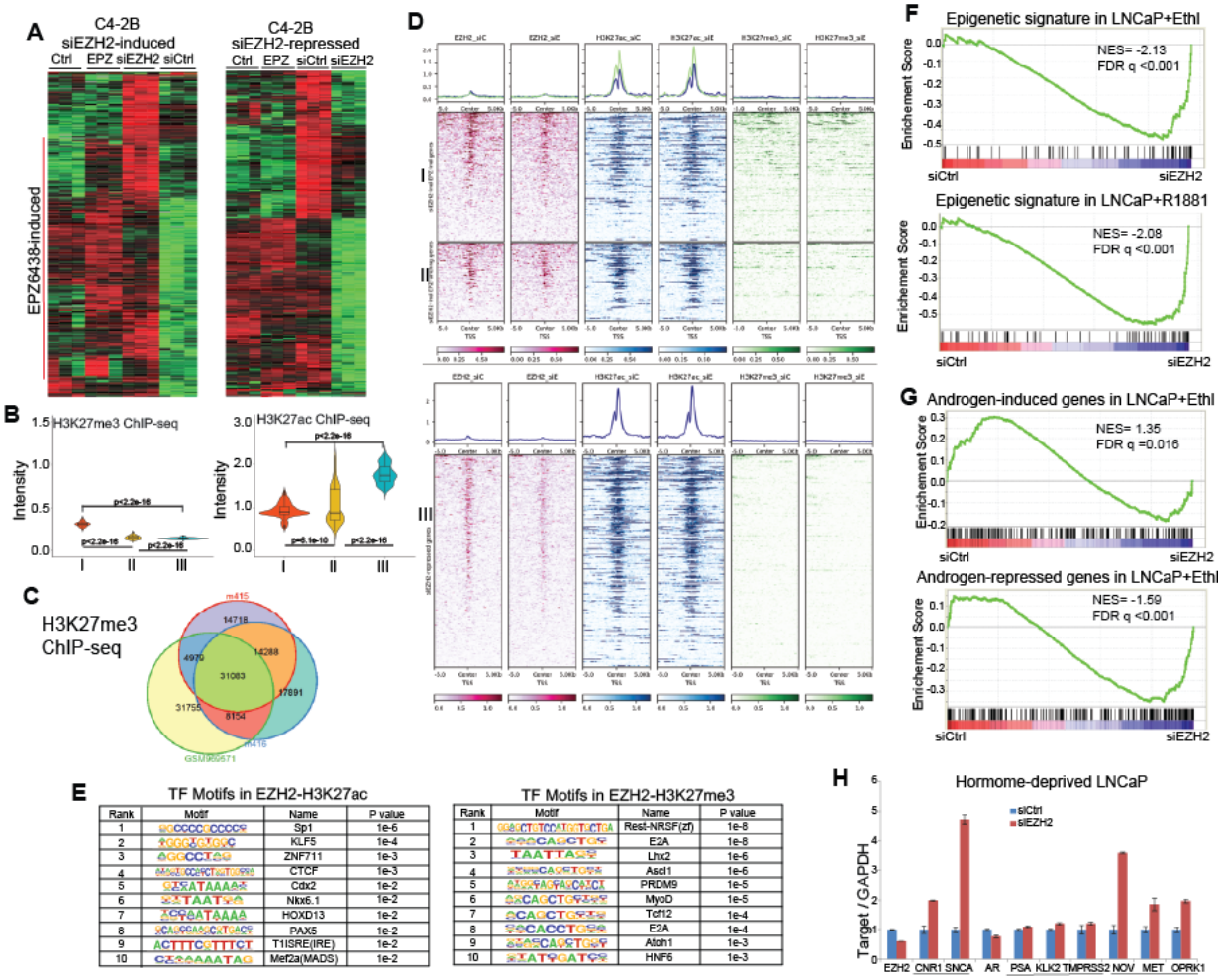

Figure S5. Dual roles of EZH2 in prostate cancer, related to Figure 5. (A) siEZH2-induced (left panel) or -repressed genes (right panel) were identified through analysis of microarray data profiling C4-2B cells with control (siCtrl) or EZH2 knockdown (siEZH2). Their expression levels in all samples including additional C4-2B cells treated with DMSO control (Ctrl) or EPZ6438 (EPZ) were clustered and visualized by heatmaps. (**B)** Boxplots showing EZH2, H3K27ac, and H3K27me3 ChIP-seq intensity for the 3 classes of EZH2-regulated genes identified in LNCaP as shown in **Fig.5A**. (**C)** Venn Diagram comparing H3K27me3 ChIP-seq performed in our lab (m415 and m416) with that presented in Xu et al. 2012 Science paper (GSM969574). (**D)** Average intensity plots and heatmaps showing EZH2, H3K27ac, and H3K27me3 ChIP-seq signals around the 3 classes of EZH2-regulated genes identified in **Fig.5A**. ChIP-seq was performed in LNCaP cells with control (siC) and EZH2 (siE) knockdown. (**E)** *De novo* motif analysis of EZH2-H3K27ac or EZH2-H3K27me3 genes that were identified in **Fig.5D**. (**F).** Epigenetic signature were obtained as genes (94 genes) that were up-regulated (adjusted p value <0.05) by EPZ-6438 as compared to control DMSO in LNCaP cells. GSEA showed that these genes are significantly enriched for up-regulation upon EZH2 knockdown (siEZH2) in both ethanol (**Upper panel**) and R1881-treated (**Lower panel**) LNCaP cells. **G.**

Androgen-induced and –repressed gene sets were obtained from microarray data of LNCaP cells as previously described (Zhao et al., 2012). GSEA showed that, in the absence of androgen (ethanol condition), androgen-induced genes (**Upper panel**) are only marginally enriched for down-regulation by siEZH2, whereas androgen-repressed genes (**Lower panel**) remained strongly enriched for up-regulation by siEZH2. **H**. LNcaP cells were hormone-starved for 3 days and subjected for control (siCtrl) or EZH2 (siEZH2) knockdown. The expression of target genes were assayed by qRT-PCR and normalized to GAPDH.

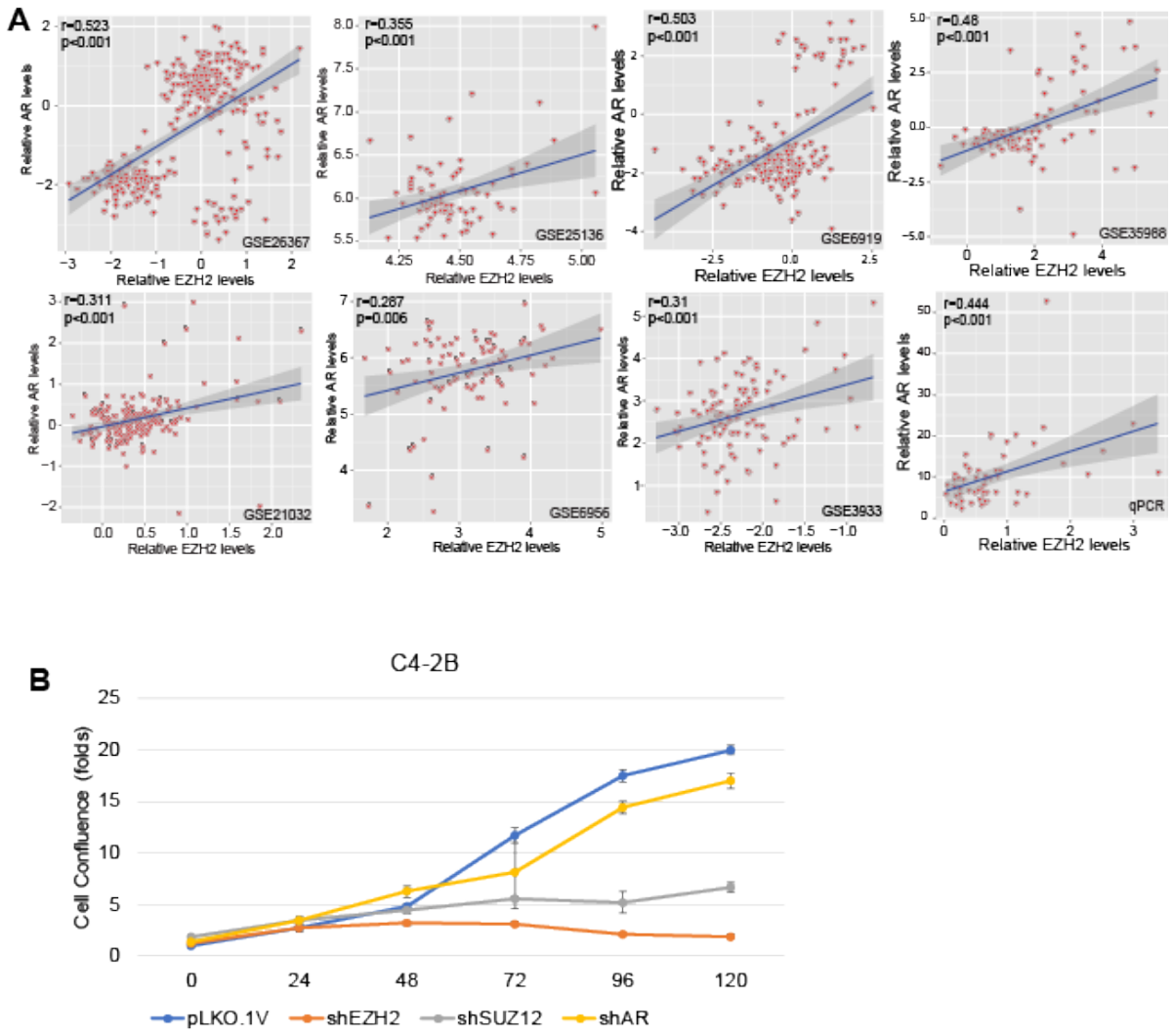

Figure S6. Targeting of prostate cancer using combinatorial EPZ6438 and Enzalutamide treatment, related to Figure 6.

(**A**) EZH2 and AR mRNA expression are positively correlated. Previously published prostate cancer gene expression data sets were downloaded from the GEO database (Ambs et al., 2008; Grasso et al., 2012; Lapointe et al., 2004; Sun and Goodison, 2009; Taylor et al.; Yu et al., 2004). The expression value of EZH2 and AR are retrieved and plotted by R ggplot2 package. Blue line represents best line of fit, and gray shades represent 95% confidence interval.

(**B**) C4-2B cells were infected with PLKO.1V, shEZH2, shSUZ12 or shAR and cell confluence were examined by Incucyte live imaging system.

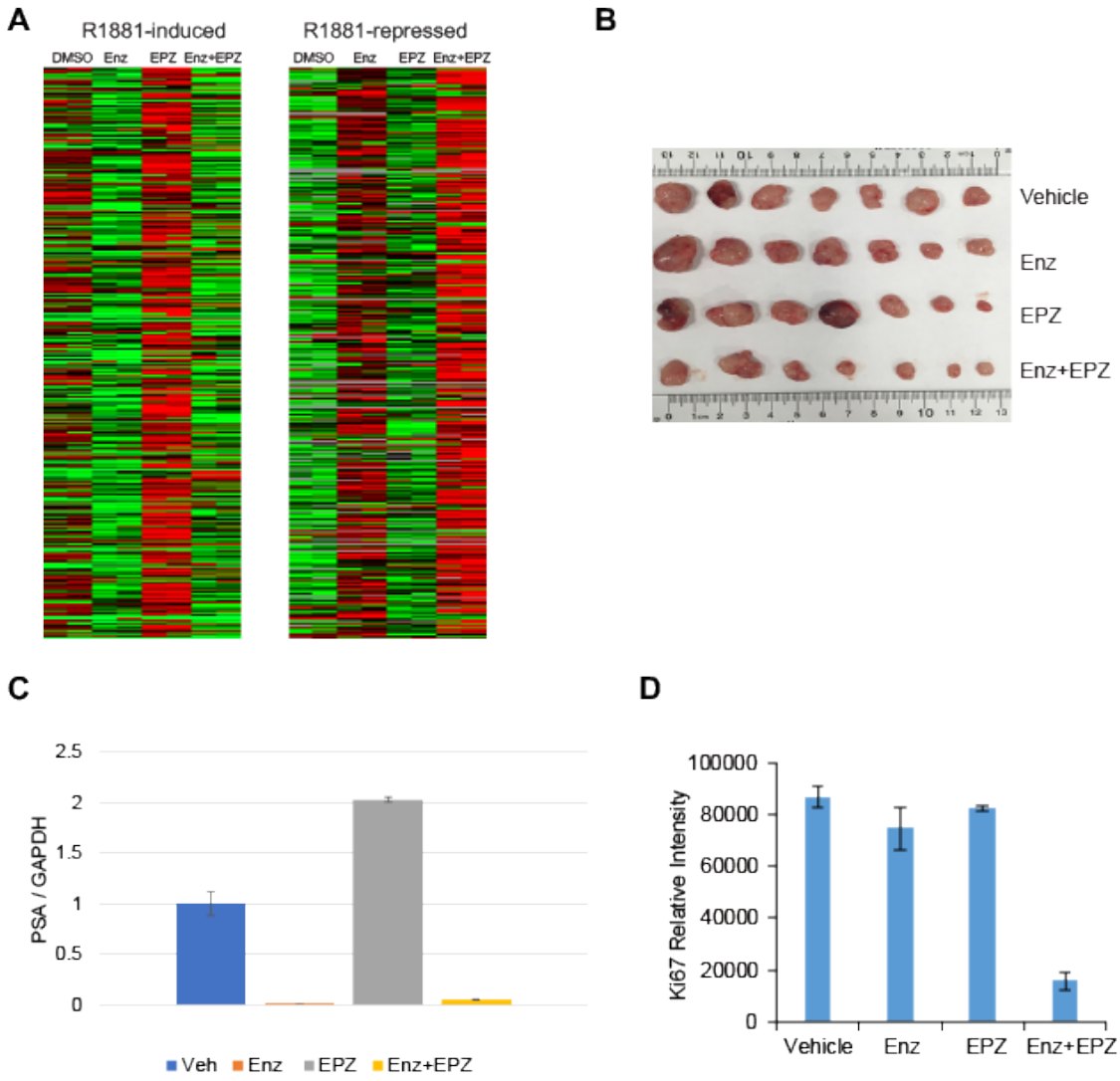

Figure S7. Combinatorial Enz and EPZ treatment inhibits AR signaling and xenograft tumor growth, related to Figure 7.

(**A**) C4-2B cells were treated with DMSO, EPZ-6438 (1μM, EPZ), enzalutamide (10μM, Enz), or both for 7 days and then subjected to RNA-seq. Experiments were done in biological triplicate. The expression (FPKM values) of androgen (R1881)-induced and androgen-repressed gene sets across all samples of varying treatments were clustered and visualized using heatmap.

(**B**) C4-2B ($2x10^6$) cells were implanted subcutaneously in surgically castrated NOD.SCID mice. Upon palpable tumor formation, the mice were randomized to receive indicated treatments (Vehicle control: 1% CMC-Na+1% tween30, 10mg/kg enzalutamide (once a day), 250mg/kg EPZ6438 (twice a day)) by oral gavage for three weeks. Xenograft tumors at the endpoint were dissected out and shown here.

(**C**) qPCR analysis of PSA from C4-2B xenograft tumors at the end points. Xengorafts from seven mice from each treatment group were used for qPCR analysis.

(**D**) Quantification of Ki-67 IHC staining. Xengorafts tissues from three mice of each group were randomly selected for Ki-67 analysis and quantification of Ki-67 was performed with Image J.

Table S5. Oligonucleotide sequences that were used in this study.

Primer Name	Sequence	Application
GAPDH F1	TGCACCACCAACTGCTTAGC	qRT-PCR
GAPDH R1	GGCATGGACTGTGGTCATGAG	qRT-PCR
EZH2 F1	TGCAGTTGCTTCAGTACCCATAAT	qRT-PCR
EZH2 R1	ATCCCCGTGTACTTTCCCATCATAAT	qRT-PCR
PSA F1	ACGCTGGACAGGGGGCAAAAG	qRT-PCR
PSA R1	GGGCAGGGCACATGGTTCACT	qRT-PCR
TMPRSS2 F1	CAGGAGTGTACGGGAATGTGATGGT	qRT-PCR
TMPRSS2 R1	GATTAGCCGTCTGCCCTCATTTGT	qRT-PCR
FKBP5 F1	TCTCATGTCTCCCCAGTTCC	qRT-PCR
FKBP5 R1	TTCTGGCTTTCACGTCTGTG	qRT-PCR
AR F1	CAGTGGATGGGCTGAAAAAT	qRT-PCR
AR R1	GGAGCTTGGTGAGCTGGTAG	qRT-PCR
ARV7 F1	CAGGGATGACTCTGGGAGAA	qRT-PCR
ARV7 R1	GCCCTCTAGAGCCCTCATTT	qRT-PCR
SLIT2 F1	CCGCAACACCGAGAGACT	qRT-PCR
SLIT2 R1	CCTGGAATGCTCCTCTTTCA	qRT-PCR
CNR1 F1	AGGAGTAAGGACCTGCGACA	qRT-PCR
CNR1 R1	TCTTGACCGTGCTCTTGATG	qRT-PCR
AR -0.7 pF1	GGGTGATTTTGCCTTTGAGA	ChIP-PCR
AR -0.7 pR1	CTGCCTTTCTTCCTGTCTGG	ChIP-PCR
AR +0.5 pF1	GCCCGAGTTTGCAGAGAG	ChIP-PCR
AR +0.5 pR1	AGTCGCCTGGCTCCTAA	ChIP-PCR
AR +1.0 pF1	TCCCGCAAGTTTCCTTCTC	ChIP-PCR
AR +1.0 pR1	GGCTGAATCTTCCACCTACTT	ChIP-PCR
AR +1.4 pF1	CACAGGCTACCTGGTCCT	ChIP-PCR
AR +1.4 pR1	TCTGGGACGCAACCTCT	ChIP-PCR
AR +1.7 pF1	CCACTTCCTCCAAGGACAATTA	ChIP-PCR
AR +1.7 pR1	CTGGACTCAGATGCTCCAAC	ChIP-PCR
AR +2.1 pF1	AAGGGCTAGAAGGCGAGA	ChIP-PCR
AR +2.1 pR1	GACTTGTAGAGAGACAGGGTAGA	ChIP-PCR
AR +2.6 pF1	TTCTGGGTCACCCTCAGC	ChIP-PCR
AR +2.6 pR1	CACCACCACCACACGGT	ChIP-PCR
AR +3.3 pF1	GTAGTTGCTTGGGTCGGTTT	ChIP-PCR
AR +3.3 pR1	CTGATGCAAACCTGAAGTAGGG	ChIP-PCR
SLIT2 pF1	CCAAGTTCATCCTTGGGAGA	ChIP-PCR

SLIT2 pR1	AAGGCAGTAGAGCCCACTCA	ChIP-PCR
KIAA0066 pF1	CTAGGAGGGTGGAGGTAGGG	ChIP-PCR
KIAA0066 pR1	GCCCCAAACAGGAGTAATGA	ChIP-PCR
pRetroX-Tight-Pur-Luc-pF	TATCCAGCCCTCACTCCTT	ChIP-PCR
0.4-0.8kb pR	AGTCGCCTGGCTCCTAA	ChIP-PCR
0.8-1.2kb pR	GGCTGAATCTTCCACCTACTT	ChIP-PCR
1.2-1.6kb pR	TCTGGGACGCAACCTCT	ChIP-PCR
1.6-1.8kb pR	CACTGCCTTACACAACTCCTTG	ChIP-PCR
1.8-2.3kb pR	GACTTGTAGAGAGACAGGGTAGA	ChIP-PCR
1.1-1.7kb-luc F	TACTCGAGGGATGGAAGTGCAGTTAGGG	cloning
1.1-1.7kb-luc R	CGCAAGCTTGCCTTCGGATACTGCTTCCT	cloning
1.7-2.5kb-luc F	CGCCTCGAGCGACCATTTCTGACAACGC	cloning
1.7-2.5kb-lucR	ACTAAGCTTCCTTCTTCGGCTGTGAAGAG	cloning
1.1-2.5kb-luc F	TACTCGAGGGATGGAAGTGCAGTTAGGG	cloning
1.1-2.5kb -lucR	ACTAAGCTTCCTTCTTCGGCTGTGAAGAG	cloning
EZH2 WT F	ATGGGCCAGACTGGGAAGAAATC	cloning
EZH2 WT R	TCAAGCGTAATCTGGAACATCGTATGGGTAAGCGTAATCTGGAACATCGTATGGGTAAGGGATTTCCATTTCTCTTTC	cloning
siEZH2 (CDS)	AAGAGGUUCAGACGAGCTGAU	knockdown
siEZH2-5'UTR	CGGTGGGACTCAGAAGGCATT	knockdown
shEZH2	CCGGGCTAGGTTAATTGGGACCAAACTCGAGTTTGGTCCCAATTAACCTAGCTTTTTG	knockdown
shSUZ12	AAGCTGTTACCAAGCTCCGT	knockdown
shAR	CCCAAGATCCTTTCTGGGA	knockdown
0.4-0.8kb F	cgcagatctCCGAGTTTGCAGAGAGGTAAC	Cloning
0.4-0.8kb R	cgcggatccAAACAAAATCCTCCACCTTCC	Cloning
0.8-1.2kb F	cgcagatctGAGACAGACTGTGAGCCTAG	Cloning
0.8-1.2kb R	cgcggatccGGAACAGATTCTGGAAAGCTC	Cloning
1.2-1.6kb F	cgcagatctTTTCCAGAATCTGTTCCAGAG	Cloning
1.2-1.6kb R	cgcggatccCAGGATGTCTTTAAGGTCAG	Cloning
1.6-1.8kb F	cgcagatctCTGACCTTAA AGACATCCTG	Cloning
1.6-1.8kb R	cgcggatccCACTGCCTTACACAACTCCTTG	Cloning
1.8-2.3kb F	cgcagatctCAAGGAGTTGTGTAAGGCAGTG	Cloning
1.8-2.3kb R	cgcggatccAGAGAGTGTGCCAGGATGAG	Cloning
EZH2 H689A F	GTTTGGATTTACCGAAGCATTTGCAAAACGAATTTTGTTACCCTTGCG	Mutagenesis
EZH2 H689A R	CGCAAGGGTAACAAAATTCGTTTTGCAAATGCTTCGGTAAATCCAAAC	Mutagenesis
sgRNA1-F	GCGGGGTAAGGGAAGTAGGG	CRISPR

sgRAN1-R	CCCTACTTCCCTTACCCCGC	CRISPR
sgRNA2-F	GCCGCCGTGGCCGCCAGCAA	CRISPR
sgRNA2-R	TTGCTGGCGGCCACGGCGGC	CRISPR
sgRNA3-F	GCCCCCTAAGTAATTGTCCTTGG	CRISPR
sgRNA3-R	CCAAGGACAATTACTTAGGGGGC	CRISPR
sgRNA4-F	AGTTGTAGTAGTCGCGACTCTGG	CRISPR
sgRNA4-R	CCAGAGTCGCGACTACTACAACT	CRISPR
sgRNA5-F	TGCATGTACGCCCCACTTTTGGG	CRISPR
SgRNA5-R	CCCAAAAGTGGGGCGTACATGCA	CRISPR
Primer-F2	CGCAAGTTTCCTTCTCTGGA	CRISPR/Cas 9 DNA PCR and RT-PCR
Primer-R2	TCCCCTTCTCTTGCTCAGAA	CRISPR/Cas 9 DNA PCR and RT-PCR