Supplemental Information

Targeting FOXA1-mediated Repression of TGF-β Signaling Suppresses Castration-Resistant Prostate Cancer Progression

Bing Song[1], Su-Hong Park[1], Jonathan C. Zhao[1], Ka-wing Fong[1], Shangze Li[1], Yongik Lee[1], Yeqing A. Yang[1], Subhasree Sridhar[1], Xiaodong Lu[1], Sarki A. Abdulkadir[2], Robert L. Vessella[3], Colm Morrissey[3], Timothy Kuzel[1,2], William Catalona[2], Ximing Yang[2], Jindan Yu[1,2,4]

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Supplemental Experimental Procedure

Plasmids, small interfering RNA and Lentiviral infections

FOXA1 stable knockdown pGIPZ lentiviral shRNAmir construct (Clone ID #V2LHS_16780) was obtained from Open Biosystems (pittsburg, PA, USA). SBE-Luc was a gift from Bert Vogelstein (Addgene plasmid # 16527).

Antibodies and Western Blots

Antibodies used in the western blots are GAPDH (Proteintech, 60004-1-Ig); pSMAD2 (Cell Signaling, 3108S); SMAD2 (Cell Signaling, 5339S); PSA (Cell Signaling, 2475S); E-Cadherin (Cell Signaling, 3195S). All antibodies were diluted as suggested by the manufacturers. Western blot analyses were performed using standard protocols. In brief, cell lysates were dissolved in 1XNP40 sample buffer, sonicated and quantified, then boiled with 1XSDS loading dye for 10 min at 95 °C, separated on a 10% SDS-polyacrylamide gel and transferred to an Amersham Hybond PVDF membrane. The membranes were blocked with 5% w/v BSA or milk in TBST for 1h at RT, incubated in primary antibody diluted in blocking solution overnight at 4°C, washed 3 × with TBST and incubated for 1 h in a secondary antibody (1:5000). Membranes were washed 3 × with TBST and incubated with ECL (GE Healthcare) for 2 min. Chemiluminescence was detected by film (GE Healthcare).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described[1]. Antibodies used include FOXA1 (Abcam, ab23738); RNA PolII p-Ser5 (Millipore, 04-1572); H3K4me3 (Millipore, 04-745).

Lentiviral Infection

The pGIPZ lentiviral shRNAmir targeting FOXA1 (Clone ID# V2LHS_16780) and control vector were purchased from Open Biosystems. The pLKO.1 lentivral shRNA targeting FOXA1 3'UTR (Clone ID# NM_004496.2-2060s21c1) were purchased from Sigma. For production of lentivirus, HEK293T cells were transfected by the PEI transfection method (Sigma) using 2ug of different lentiviral vector, 1.5ug of psPAX2 and 0.5ug of pMD2G. Supernatant containing lentiviruses was harvested at 48h after transfection and filtered through a 0.45 um filter. Lentiviruses, supplemented with 8ug/ml polybrene, were used to infect cells. 48h after infection, cells were selected with 2ug/ml puromycin.

Cell Proliferation assay

Proliferation assay was performed using WST-1 (Clontech Laboratories) as described by the manufacturer. In brief, 50,000 C4-2B cells/well and 100,000 VCaP cells/well were seeded in 24 wells plate. The cells were either treated with vehicle (DMSO) or 10μM Enz and/or 10μM LY2157299, 50μL of WST-1 was added to the cultures and incubated for 2 hours when harvesting, and the absorbance was measured at 440nm using a spectrophotometer.

RNA Isolation and Quantitative RT-PCR

Total cellular RNAs were isolated using the Invitrogen Trizol reagent. For cDNA synthesis, 500ng of total RNA were reverse transcribed with qScript cDNA SuperMix (Quanta BioSciences). qRT-PCR analysis was done using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Results were analyzed using StepOne Software v2.1 (Applied Biosystems), and the relative expression of mRNA was determined using GAPDH as the loading control. Data are from triplicate samples.

Immunofluorescence Staining

Control and FoxA1 knockdown cells were grown on coverslips in 6-well plates and fixed with 4% PFA in PBS for 10 mintues, permeabilized with methanol for 2 minutes at room temperature, rinsed in PBS+0.1%TritonX-100, and blocked with 3% BSA for 5 minutes in a humidified chamber. After fixing and blocking, coverslips were labeled with 100μL primary antibody overnight in the cold room. Primary antibody FoxA1 (1:400 dilution, sc101058, Santa Cruz); E-Cadherin (1:200 dilution, 3195S, Cell Signaling) were used for immunostaining. The coverslips were washed and labeled with 100μL of secondary antibody conjugated with Alexa 594 or Alexa 488, (1:400 dilution, A-11037/11034, Invitrogen) for 60 minutes. After PBS washes, coverslips were mounted with 30 μL ProLong Gold Antifade Reagent (P36930, Invitrogen). A fluorescence microscope from the Northwestern (Nikon A1 Confocal Laser Microscope System) was used in imaging.

Immunohistochemistry of mouse xenograft tissues

IHC was performed on paraffin-embedded VCaP tumor xenograft. After deparaffinization, rehydration and antigen retrieval with citrate acid at pH6, tissues were permeabilized with 0.5% Triton X-100, peroxidase blocked with 0.3% H_2O_2, blocked with avidin and biotin, followed by 2% BSA protein block. Then, slides were incubated with anti-ki67 (1:400, D3B5, Cell Signaling) or anti-pSMAD2 (1:2000, AB3849, Chemicon) for 1h at RT, washed 5X with TBST and incubated with biotin-conjugated secondary antibody (1:200) for 15 min at RT. After extensive washing with TBST, slides were incubated with streptavidin-HRP (1:500) for 15 min at RT, wash 3X with TBST and incubated with DAB substrate for 5-10 min at RT until desired color intensity was reached. After that, slides were counterstained with hematoxylin for 1s, extensive washed with water, dehydrated in ethanol, cleared with Xylene and finally mounted

by permount. Images of IHC were taken and the percentage of cells with positive staining were quantified using ImageJ. Briefly, a random field from each tumor section was counted. First, a field with the greatest cell intensities was chosen to calculate an arbitrary threshold that was applied to the rest of the samples. After setting the separate thresholds for pSMAD2 and Ki67 staining, any cells that met the cutoff threshold were counted as pSMAD2-positve or Ki67-positive cells. Four random fields from each of the two slides for each genotype were counted. The number of pSMAD2-positive or Ki67-positive cells were counted manually using the cell counter plugin in ImageJ. The results are presented as percentage of pSMAD2-positive or Ki67-positive cells among the total cell population in a given field view.

Tissue Microarray analysis

After deparaffinization, rehydration and antigen retrieval with citrate acid at pH6, tissues were permeabilized with 0.5% Triton X-100, peroxidase blocked with 0.3% H_2O_2, blocked with avidin and biotin, followed by 2% BSA protein block. Human TMA immunohistochemical staining was conducted using the Dako Autostainer Link 48 with enzyme labeled biotin streptavidin system and solvent-resistant DAB Map kit. Antibodies used in IHC include anti-FoxA1 (1:400, ab23738, Abcam) and anti-pSMAD2 (1:2000, AB3849, Chemicon). Images were captured with use of TissueGnostics, exported to TissueFAXs viewer and analyzed using Photoshop CS4 (Adobe). The quantification of IHC uses a score of 0 to 3 for intensities of negative, weak, moderate and strong.

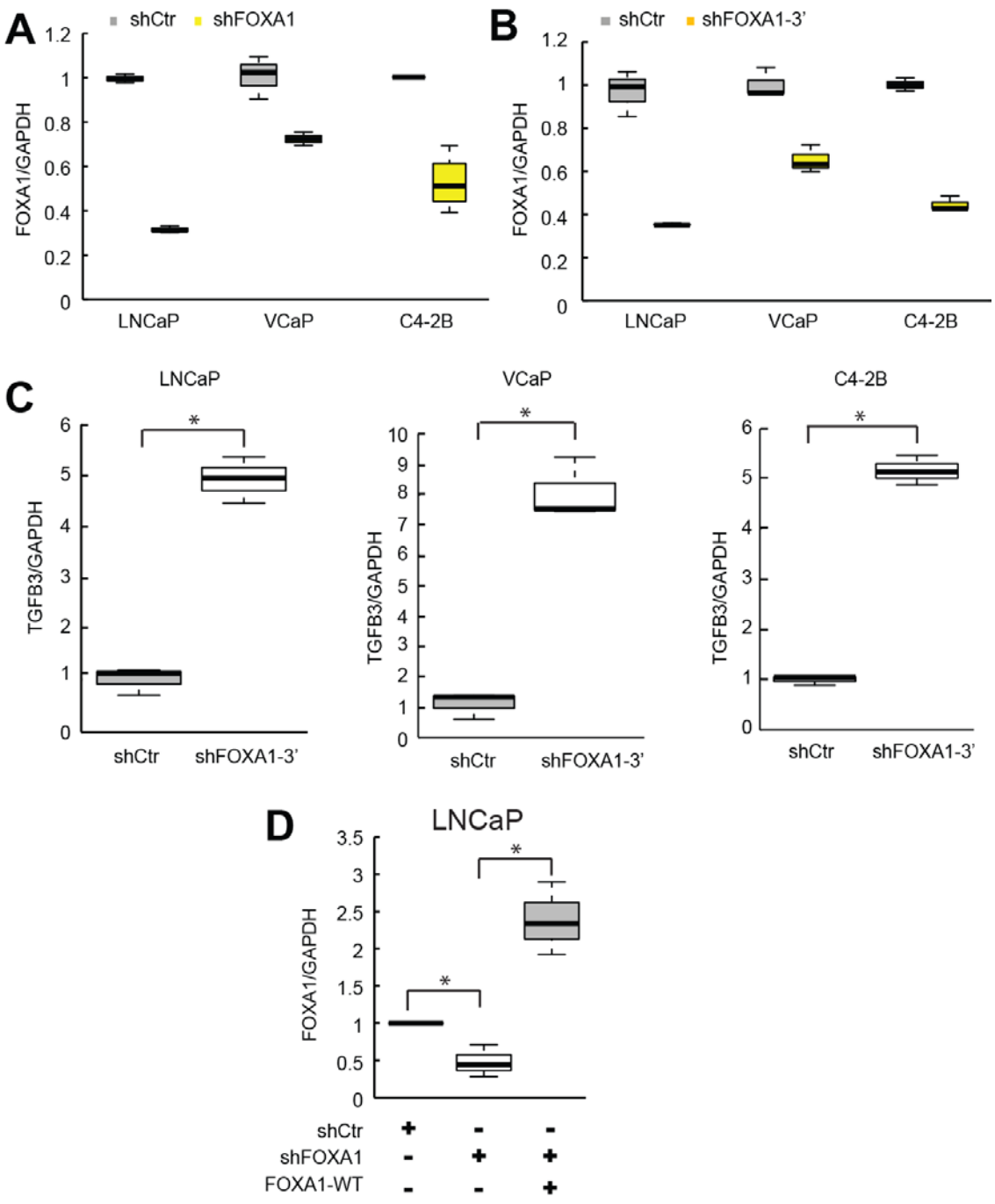

Figure S1. FOXA1 represses *TGFB3* gene transcription. (A,B) qRT-PCR analysis of FOXA1 gene expression in shCtr vs. shFOXA1 (**A**) and shCtr vs. shFOXA1-3' (**B**) LNCaP, VCaP, and C4-2B cells. Data normalized to GAPDH. **(C)** *TGFB3* gene expressions upregulated upon FOXA1 knockdown using the second shRNA (shFOXA1-3'). **(D)** FOXA1-WT overexpression rescues FOXA1 loss in LNCaP cells infected with shFOXA1 knockdown lentivirus. Cells were harvested for qRT-PCR analysis. (n=3, *p<0.05).

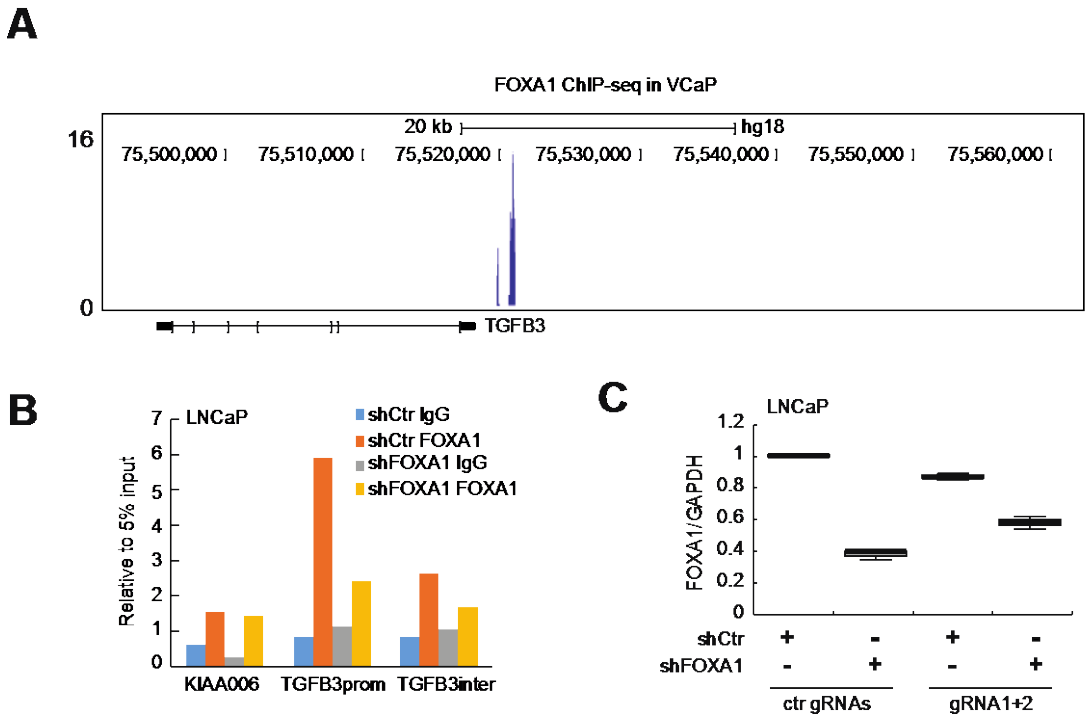

Figure S2. FOXA1 binds to *TGFB3* enhancer to repress its expression. (A) Genome browser view showing FOXA1 binding at the *TGFB3* enhancer. FOXA1 ChIP-seq was performed in VCaP cells. **(B)** Zoom-in of the three left panels in **Figure 2B**. FOXA1 and IgG ChIP were performed in shCtrl and shFOXA1 LNCaP cells and subjected to qPCR using primers flanking the promoter (prom), enhancer (enh) and an intermediate (inter) region (as a negative control) of *TGFB3* gene. *KIAA0066* was used as a negative control (n=2). **(C)** qRT-PCR analysis of *FOXA1* gene expression in LNCaP cells treated with CRISPR/Cas9 with control sgRNAs (ctr) or TGFB3 enhancer-targeting sgRNAs (gRNA1+2).

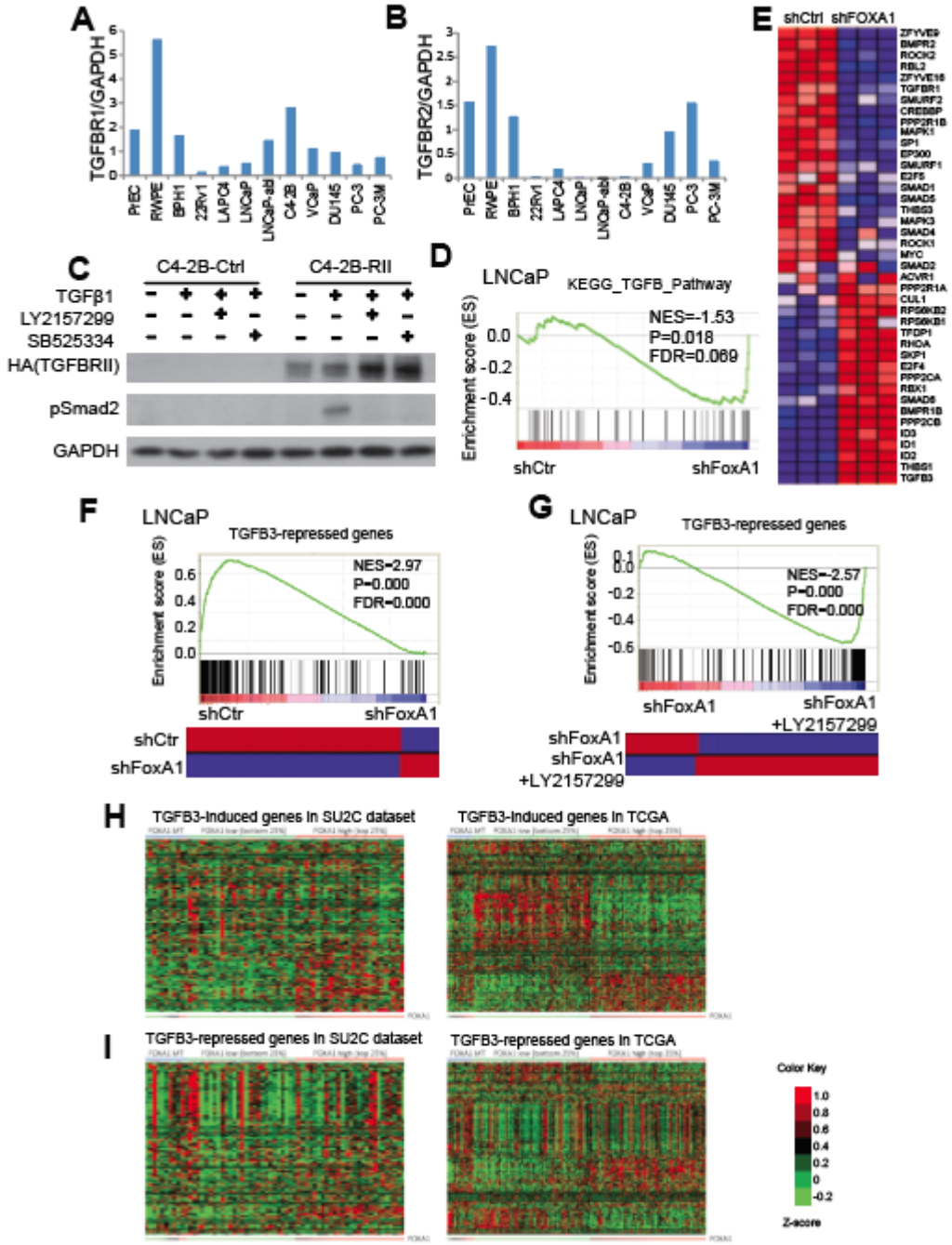

Figure S3. TGF-beta pathway genes are enriched for regulation by FOXA1. (A-B) TGFBR1 and TGFBR2 expression levels in various prostate cell lines were examined by qRT-PCR (n=2). **(C)** C4-2B-Ctrl and C4-2B-RII stable cells were treated with DMSO or 5ng/ml TGF-β1 ligand for 4 days, with or without subsequent 10uM LY2157299 treatment for 1 day. Cells were then collected and analyzed by western blotting. **(D,E)** GSEA (**D**) and heat map analysis (**E**) for KEGG_TGFB_pathway genes showing enriched expression in FOXA1-knockdown (shFOXA1) cells compared to control LNCaP cells (shCtr). **(F-G)** GSEA and heatmap analysis of TGFB3-repressed genes showing enrichment for down-regulation by FOXA1 depletion (**F**) but rescue by LY2157299 (**G**). (**H**) Heatmap of the TGF-β3-induced gene expressions (mRNA expression z-scores) for FOXA1 mutant, FOXA1-low and FOXA-high subgroups of SU2C and TCGA samples. TGF-β3-induced genes were identified using

cutoff of ≥2.5 fold change with at least one sample p value < 0.001. (**I**) Heatmap of the TGF-β3-repressed gene expressions (mRNA expression z-scores) for FOXA1 mutant, FOXA1-low and FOXA-high subgroups of SU2C and TCGA samples. TGF-β3-repressed genes were identified using cutoff of ≥2 fold change with at least one sample p value < 0.001.

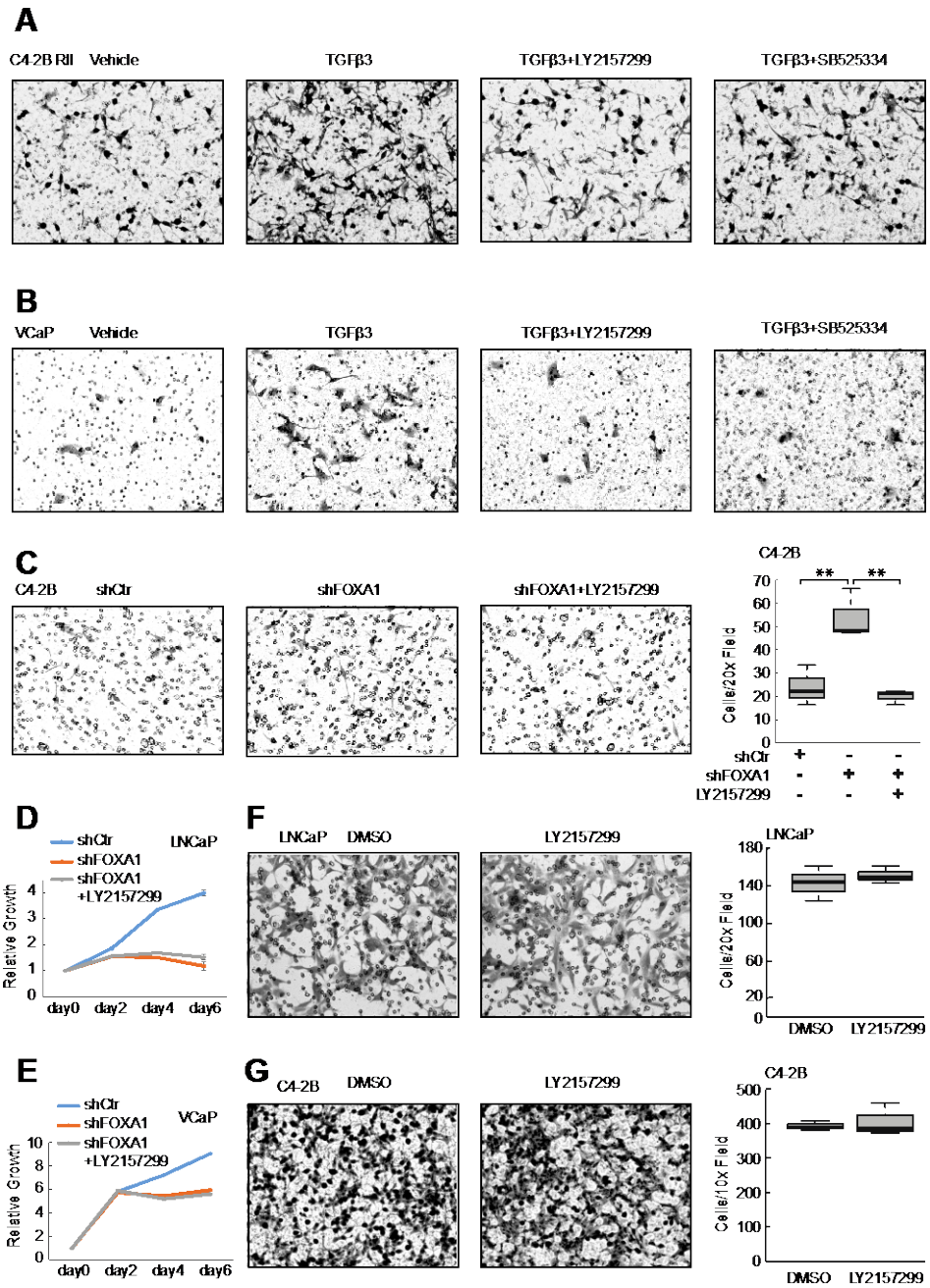
A
C4-2B RII Vehicle
TGFβ3
TGFβ3+LY2157299
TGFβ3+SB525334
B
VCaP Vehicle
TGFβ3
TGFβ3+LY2157299
TGFβ3+SB525334
C
C4-2B shCtr
shFOXA1
shFOXA1+LY2157299
C4-2B
Cells/20x Field
shCtr
shFOXA1
LY2157299
D
LNCaP
shCtr
shFOXA1
shFOXA1 +LY2157299
Relative Growth
day0 day2 day4 day6
E
VCaP
shCtr
shFOXA1
shFOXA1 +LY2157299
Relative Growth
day0 day2 day4 day6
F
LNCaP DMSO
LY2157299
LNCaP
Cells/20x Field
DMSO LY2157299
G
C4-2B DMSO
LY2157299
C4-2B
Cells/10x Field
DMSO LY2157299
H
Merge
DAPI
E-Cadherin
FOXA1
shCtr
shFOXA1
shFOXA1 + LY2157299

Figure S4. LY2157299 abolishes cell invasion caused by TGF-β3 stimulation or FOXA1 knockdown. (A,B) Matrigel invasion assay of C4-2B-RII **(A)** and VCaP **(B)** cells treated with 5ng/ml TGF-β3 ligand with or without 10μM LY2157299 or 5μM SB525334. **(C)** Matrigel invasion assay of C4-2B shCtr or shFOXA1 stable cells treated with or without 10μM LY2157299. The number of invaded cells per 20x objective field was counted from 3 fields per conditions (n=3, *$p<0.05$, 1-way ANOVA with Tukey post-hoc test). **(D,E)** WST-1 assays of LNCaP **(D)** and VCaP **(E)** shCtr or shFOXA1 stable cells treated with or without 10μM LY2157299. (**F,G**) Matrigel invasion assay of LNCaP **(F)** and C4-2B **(G)** parental cells treated with or without 10μM LY2157299. There were no significant differences. (**H**) Immunofluorescence staining showing EMT-like changes of cell morphology upon FOXA1 knockdown in LNCaP cells, which was reversed by LY2157299 treatment. Cells were stained for DAPI, FOXA1, and epithelial marker E-Cadherin. Nuclei were stained with DAPI. Scale bar, 50um.

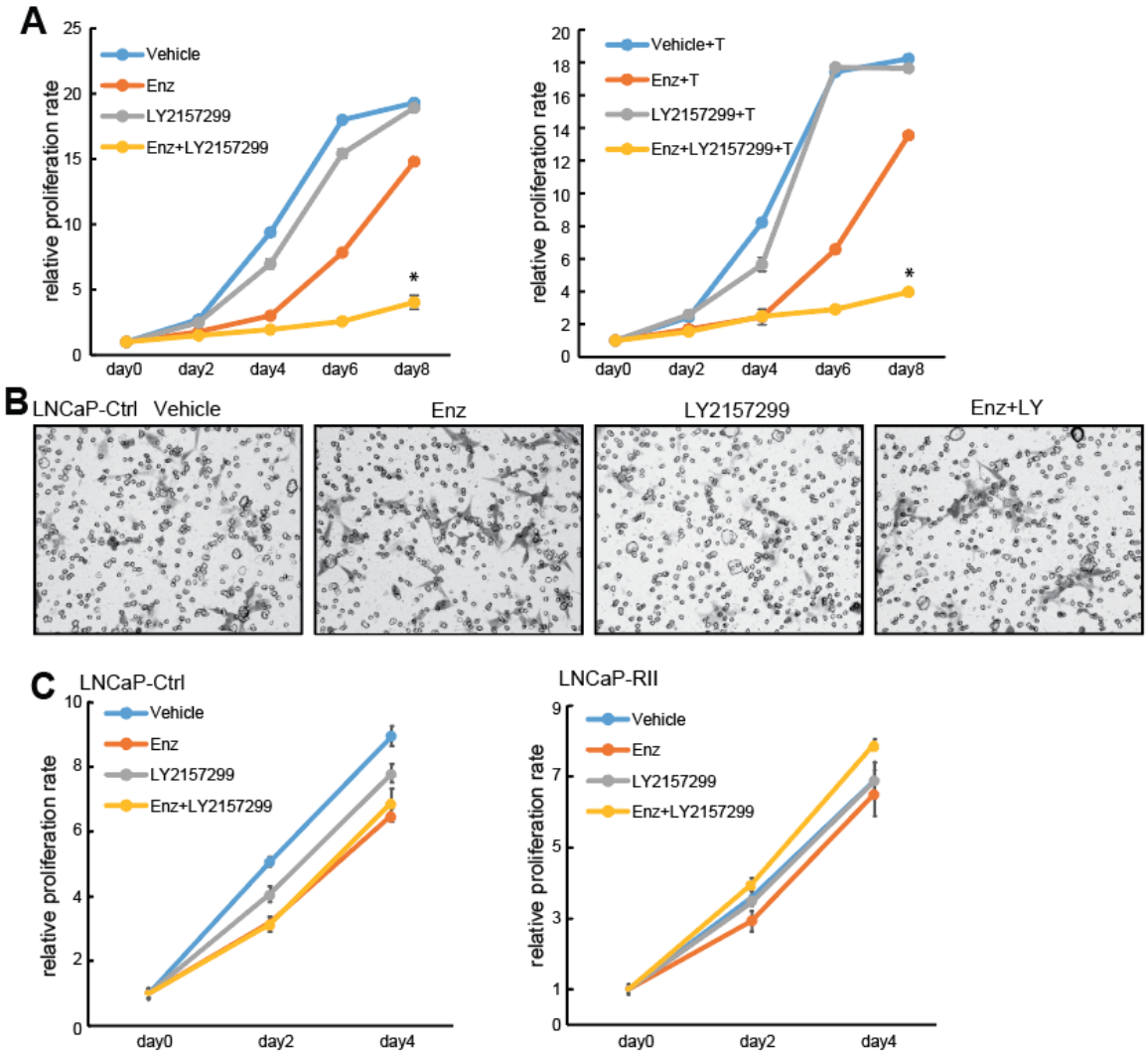

Figure S5. Enzalutamide and LY2157299 drug combination on prostate cancer cell proliferation. (A) WST-1 assays of VCaP cells that were treated with vehicle control, 10μM Enz, 10μM LY2157299, or their combination (left panel). WST-1 assays of VCaP cells treated with vehicle control, 10μM Enz, 10μM LY2157299, or their combination in the presence of 5ng/ml TGF-β3 ligand (right panel). **(B)** Matrigel assays of LNCaP-Ctrl stable cells treated with vehicle control, 10μM Enz, 10μM LY2157299, or their combination. (**C**) WST-1 assays of LNCaP-Ctrl and LNCaP-RII stable cells that were treated with vehicle control, 1μM Enz, 10μM LY2157299, or their combination.

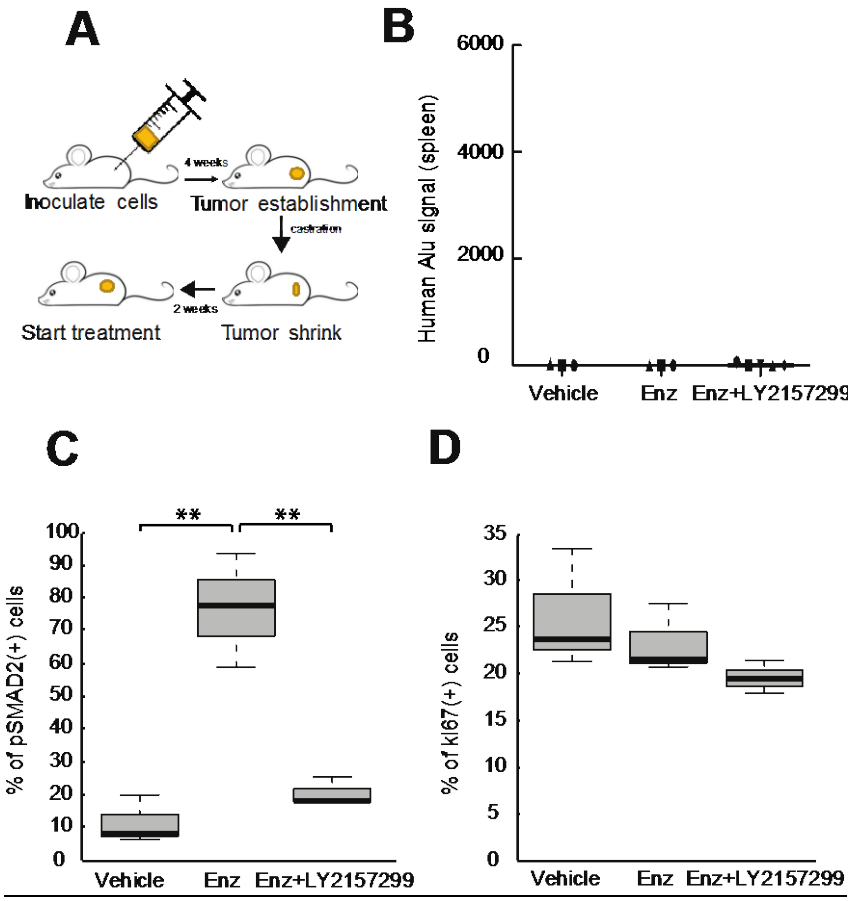

Figure S6. LY2157299 sensitizes CRPC xenograft tumors to Enz. (A) Schematics illustrating VCaP castration resistant xenograft tumor establishment and treatment. **(B)** Mice bearing VCaP xenografts were treated with vehicle, 10μM Enz alone or in combination with 10μM LY2157299 for 33 days. At the endpoint, the spleen were dissected. Genomic DNA were isolated and analyzed for metastasized cells by measuring human Alu sequence (by Alu-qPCR). (**C-D**) Immunohistochemistry was performed in tumor sections prepared from xenograft mice with pSMAD2 and Ki67 antibodies. The pSAMD2-(**C**) or Ki67-(**D**) positive cells were counted using cell counter plugin in ImageJ and the percentage of positive cells are illustrated (n=3, **p<0.01, 1-way ANOVA with Tukey post-hoc test).

Supplemental Table 1. Oligonucleotide sequences that were used in this study

Name	Sequence	Application
FOXA1 F	GAAGATGGAAGGGCATGAAA	qRT-PCR
FOXA1 R	CGCTCGTAGTCATGGTGTTC	qRT-PCR
GAPDH F1	TGCACCACCAACTGCTTAGC	qRT-PCR
GAPDH R1	GGCATGGACTGTGGTCATGAG	qRT-PCR
TGFB3 F1	AGGAGATGCATGGGGAGAG	qRT-PCR
TGFB3 R1	TTTAGGGCAGACAGCCAGTT	qRT-PCR
TGFB3 pF1	TCTCAGCCAACCAGTGTCAG	ChIP-qPCR
TGFB3 pR1	ACCACAAATTCCCAGCCTAA	ChIP-qPCR
TGFB3 pF2	GCTGCCTCTCGTCTTCATTG	ChIP-qPCR
TGFB3 pR2	TCCCTGCCTTCCAATGCATA	ChIP-qPCR
TGFB3 pF3	TCTAGGCCTTTTCCCACGAG	ChIP-qPCR
TGFB3 pR3	CCTCTCAGAAAGGGTCCAGG	ChIP-qPCR
KIAA0066 pF2	CTAGGAGGGTGGAGGTAGGG	ChIP-qPCR
KIAA0066 pR2	GCCCCAAACAGGAGTAATGA	ChIP-qPCR
TGFB3 gRNA2F	CACCG GCCTGTGACAGCAAGAGCAC	CRISPR
TGFB3 gRNA2R	AAAC GTGCTCTTGCTGTCACAGGC C	CRISPR
TGFB3 gRNA1F	CACCG GGGCATTAACAACCTTGTGT	CRISPR
TGFB3 gRNA1R	AAAC ACACAAGGTTGTTAATGCCC C	CRISPR
TGFB3 gRNA test F	TGATGCCTCCCACTTGTACA	CRISPR
TGFB3 gRNA test R	CAGAGCCCTTTCCGCTATC	CRISPR
TGFBfull_F	CTGAGCTCGCTAGCC TCGAG GAAGGGGCTTGAGGAAAATC	Cloning
TGFBfull_R	AGTACCGGATTGCCA AGCT GTTTTGCTGGGCTCTGACTC	Cloning
Human Alu Fwd	GTCAGGAGATCGAGACCATCCT	
Human Alu Rev	AGTGGCGCAATCTCGGC	

References:

1. **Jin, H.J., Zhao, J.C., Ogden, I., Bergan, R.C. & Yu, J. Androgen receptor-independent function of FoxA1 in prostate cancer metastasis. *Cancer Res* 73, 3725-3736 (2013).**