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Supplemental Information

Exploiting the tumor-suppressive activity of the androgen receptor by CDK4/6 inhibition in castration-resistant prostate cancer

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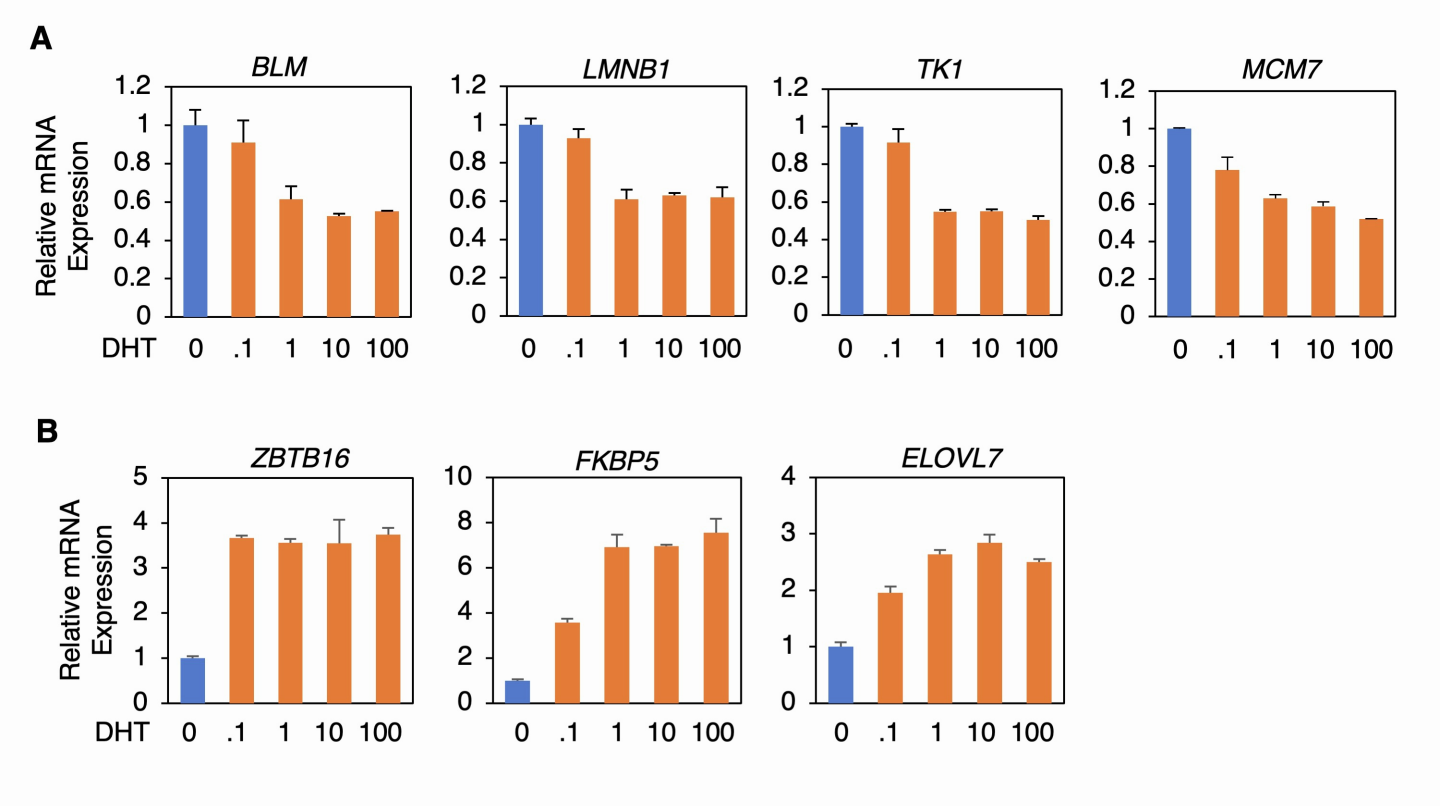
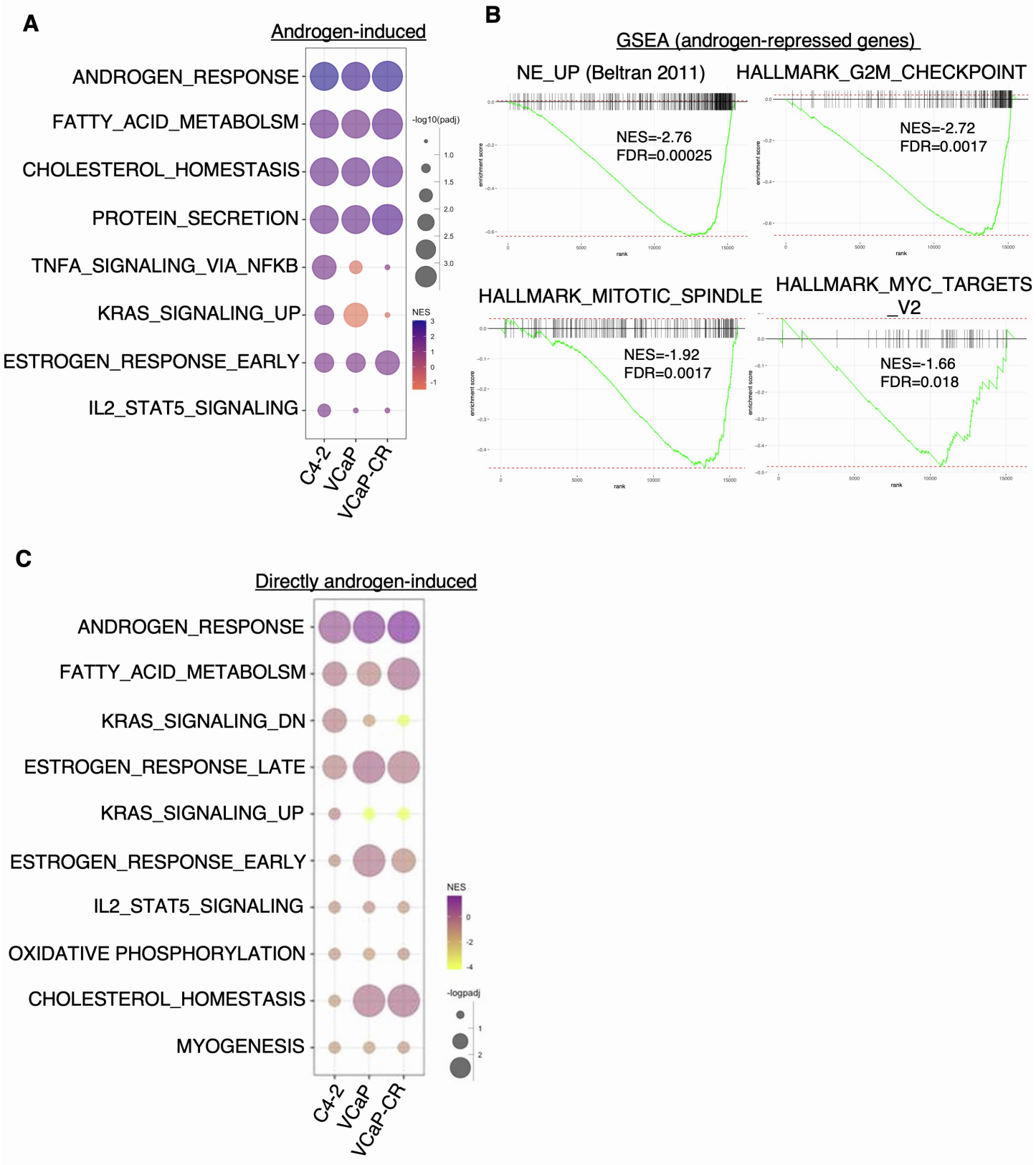
Supplementary Information Inventory:



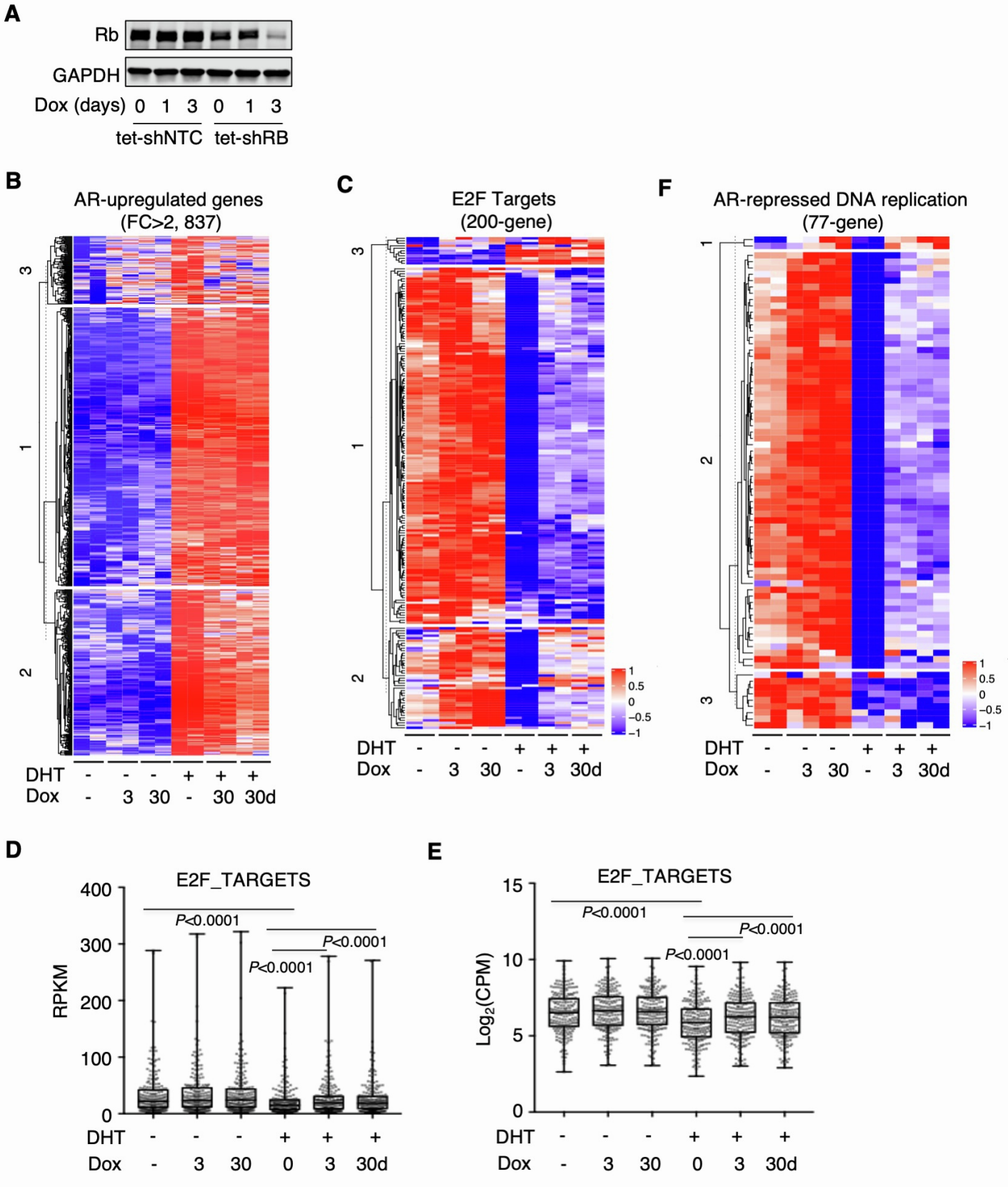
Supplementary Figure S1, related to Figure 1

(**A, B**) C4-2 cells were hormone-depleted for 3d and then treated with DHT (0-100nM) for 24h. qRT-PCR analyses were done to examine the expression of AR-repressed DNA synthesis genes (A) and classic AR-activated genes (B).


A
Androgen-induced
ANDROGEN_RESPONSE
FATTY_ACID_METABOLSM
CHOLESTEROL_HOMESTASIS
PROTEIN_SECRETION
TNFA_SIGNALING_VIA_NFKB
KRAS_SIGNALING_UP
ESTROGEN_RESPONSE_EARLY
IL2_STAT5_SIGNALING
C4-2
VCaP
VCaP-CR
-log10(padj)
1.0
1.5
2.0
2.5
3.0
NES
3
2
1
0
-1
B
GSEA (androgen-repressed genes)
NE_UP (Beltran 2011)
NES=-2.76
FDR=0.00025
HALLMARK_G2M_CHECKPOINT
NES=-2.72
FDR=0.0017
HALLMARK_MITOTIC_SPINDLE
NES=-1.92
FDR=0.0017
HALLMARK_MYC_TARGETS_V2
NES=-1.66
FDR=0.018
enrichment score
rank
C
Directly androgen-induced
ANDROGEN_RESPONSE
FATTY_ACID_METABOLSM
KRAS_SIGNALING_DN
ESTROGEN_RESPONSE_LATE
KRAS_SIGNALING_UP
ESTROGEN_RESPONSE_EARLY
IL2_STAT5_SIGNALING
OXIDATIVE PHOSPHORYLATION
CHOLESTEROL_HOMESTASIS
MYOGENESIS
C4-2
VCaP
VCaP-CR
NES
0
-2
-4
-logpadj
1
2

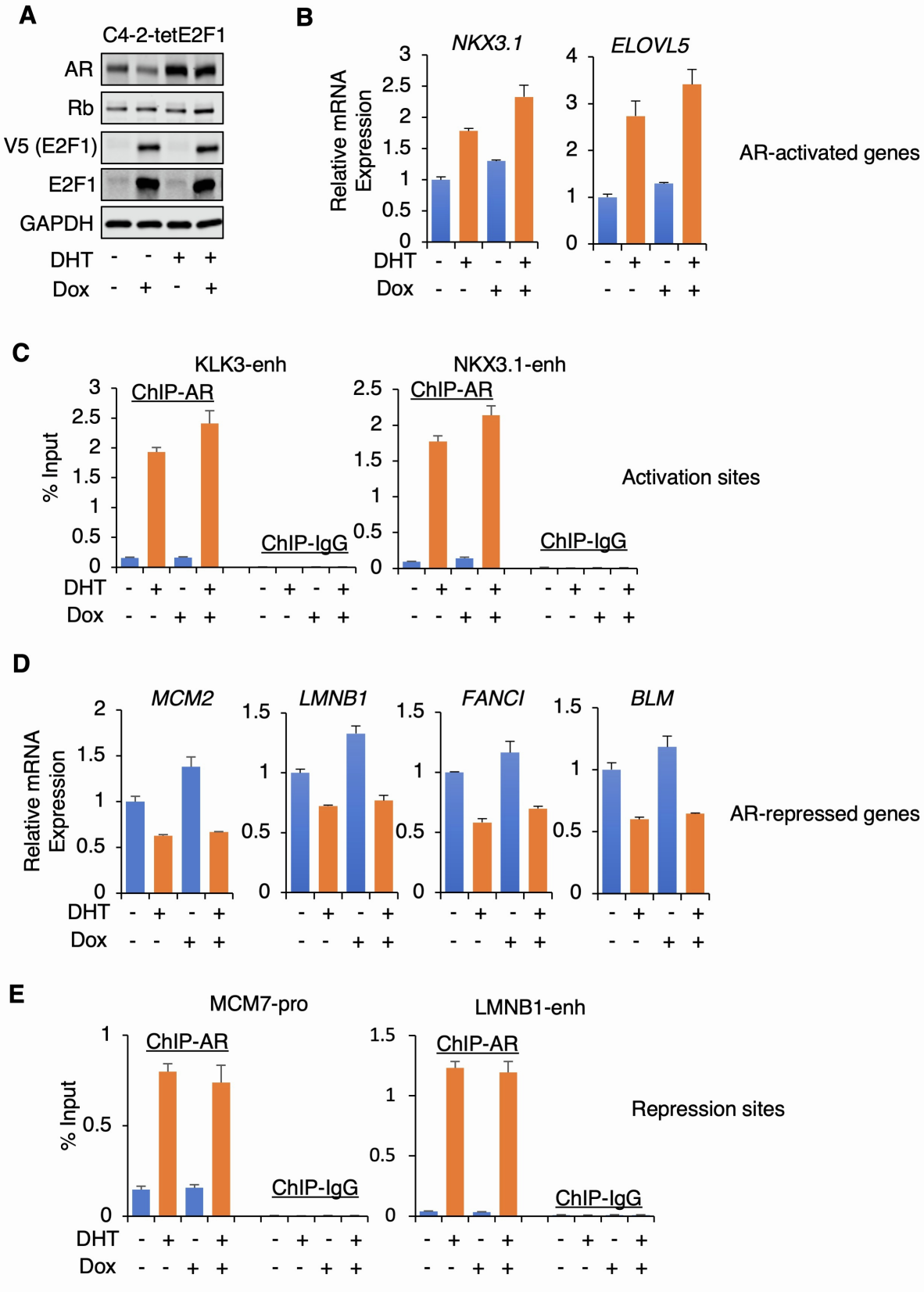
Supplementary Figure S2, related to Figure 1

(**A**) Gene Set Enrichment Analysis (GSEA) was done to compare the androgen-induced genes in C4-2-tet-shRB versus VCaP/VCaP-CR cells. (**B**) Enrichments of NE_UP,[1] HALLMARK_G2M_CHECKPOINT, HALLMARK_MITOTIC_SPINDLE, and HALLMARK_MYC_TARGETS_V2 gene sets for AR-repressed genes were plotted. (**C**) GSEA was done to compare directly androgen-induced genes (AR-activated genes with nearby AR binding) in these cells.


A
Rb
GAPDH
Dox (days) 0 1 3 0 1 3
tet-shNTC tet-shRB
B
AR-upregulated genes
(FC>2, 837)
DHT - - - + + +
Dox - 3 30 - 30 30d
C
E2F Targets
(200-gene)
DHT - - - + + +
Dox - 3 30 - 3 30d
F
AR-repressed DNA replication
(77-gene)
DHT - - - + + +
Dox - 3 30 - 3 30d
D
E2F_TARGETS
RPKM
P<0.0001
P<0.0001
P<0.0001
DHT - - - + + +
Dox - 3 30 0 3 30d
E
E2F_TARGETS
Log2(CPM)
P<0.0001
P<0.0001
P<0.0001
DHT - - - + + +
Dox - 3 30 0 3 30d

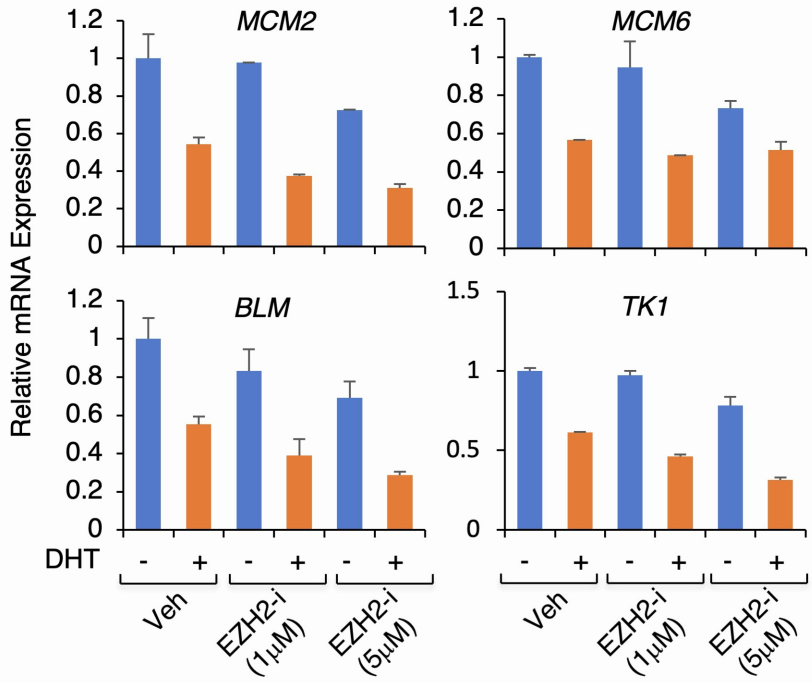
Supplementary Figure S3, related to Figure 2

(**A**) Immunoblotting for Rb in the control or C4-2-tet-shRB cells with 0.05μg/ml doxycycline for 0-3d. (**B**, **C**, **F**) Heatmap view for androgen-upregulated genes (B), E2F target genes (C), and previously identified AR repressed DNA replication genes[2] (F). (**D**, **E**) Box plots for the expression of E2F target genes calculated as RPKM (D) or Log_2(CPM) (E) in these samples. Note: RPKM is Reads Per Kilobase of transcript per Million mapped reads and CPM is Counts Per Million mapped reads.


A
C4-2-tetE2F1
AR
Rb
V5 (E2F1)
E2F1
GAPDH
DHT - - + +
Dox - + - +
B
NKX3.1
ELOVL5
Relative mRNA Expression
DHT - + - +
Dox - - + +
AR-activated genes
C
KLK3-enh
NKX3.1-enh
ChIP-AR
ChIP-IgG
% Input
DHT - + - + - + - +
Dox - - + + - - + +
Activation sites
D
MCM2
LMNB1
FANCI
BLM
Relative mRNA Expression
DHT - + - +
Dox - - + +
AR-repressed genes
E
MCM7-pro
LMNB1-enh
ChIP-AR
ChIP-IgG
% Input
DHT - + - + - + - +
Dox - - + + - - + +
Repression sites

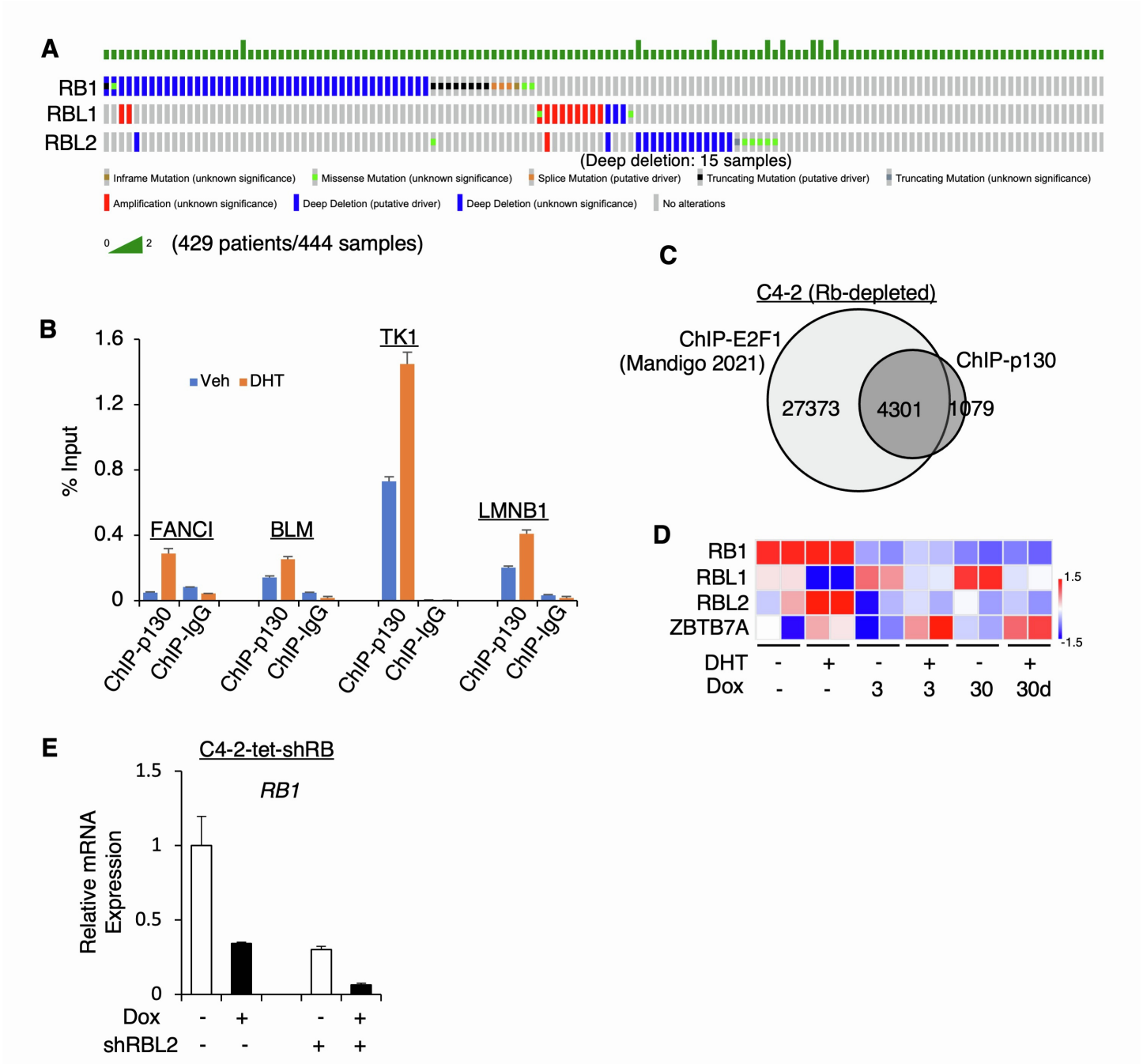
Supplementary Figure S4, related to Figure 2

(**A**) Immunoblotting for indicated proteins in C4-2-tetE2F1 cells (expressing doxycycline-regulated V5-tagged E2F1) treated with or without DHT (10nM, 24h) or doxycycline (0.25μg/ul, 3d). (**B**, **D**) qRT-PCR for AR-activated genes (B) and AR-repressed DNA synthesis genes (D). (**C**, **E**) C4-2-tetE2F1 cells were pretreated with doxycycline (3d) and/or treated with DHT (10nM, 4h). ChIP-qPCR for AR binding was examined at AR activation sites (C) or AR repression sites (E).



Supplementary Figure S5, related to Figure 2

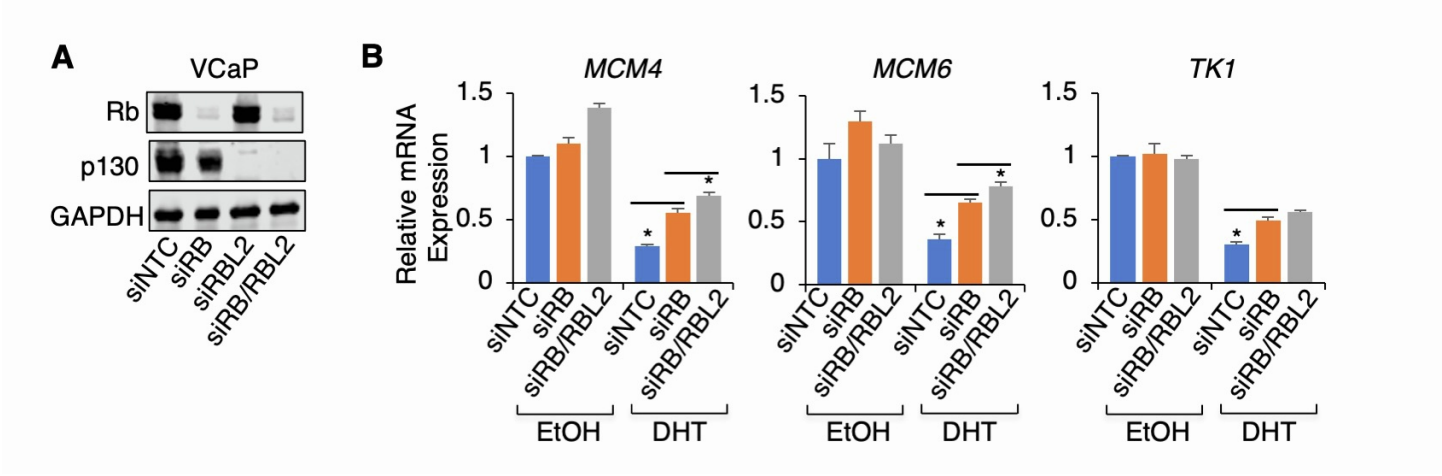
qRT-PCR for a panel of AR-repressed DNA synthesis genes in C4-2 cells treated with DHT (10nM, 24h) and EZH2 inhibitor GSK126 (0-5μM, 24h).



Supplementary Figure S6, related to Figure 3**.**

(**A**) Genomic alterations of *RB1*, *RBL1*, and *RBL2* in SU2C mCRPC dataset (data were analyzed using cBioPortal).[3-5] (**B**) ChIP-qPCR for p130 in C4-2-tet-shRB cells treated with DHT (10nM, 4h). (**C**) The Venn diagram for p130 (C4-2-tet-shRB with doxycycline) and E2F1 (C4-2 with *RB1* silencing)[6] overlapping binding peaks under Rb-depleted condition. (**D**) Heatmap view for the expression levels of *RB1*, *RBL1*, *RBL2*, and

ZBTB7A in C4-2-tet-shRB cells. (**E**) qRT-PCR for *RB1* expression in C4-2-tet-shRB cells treated with doxycycline or stably infected with shRBL2.

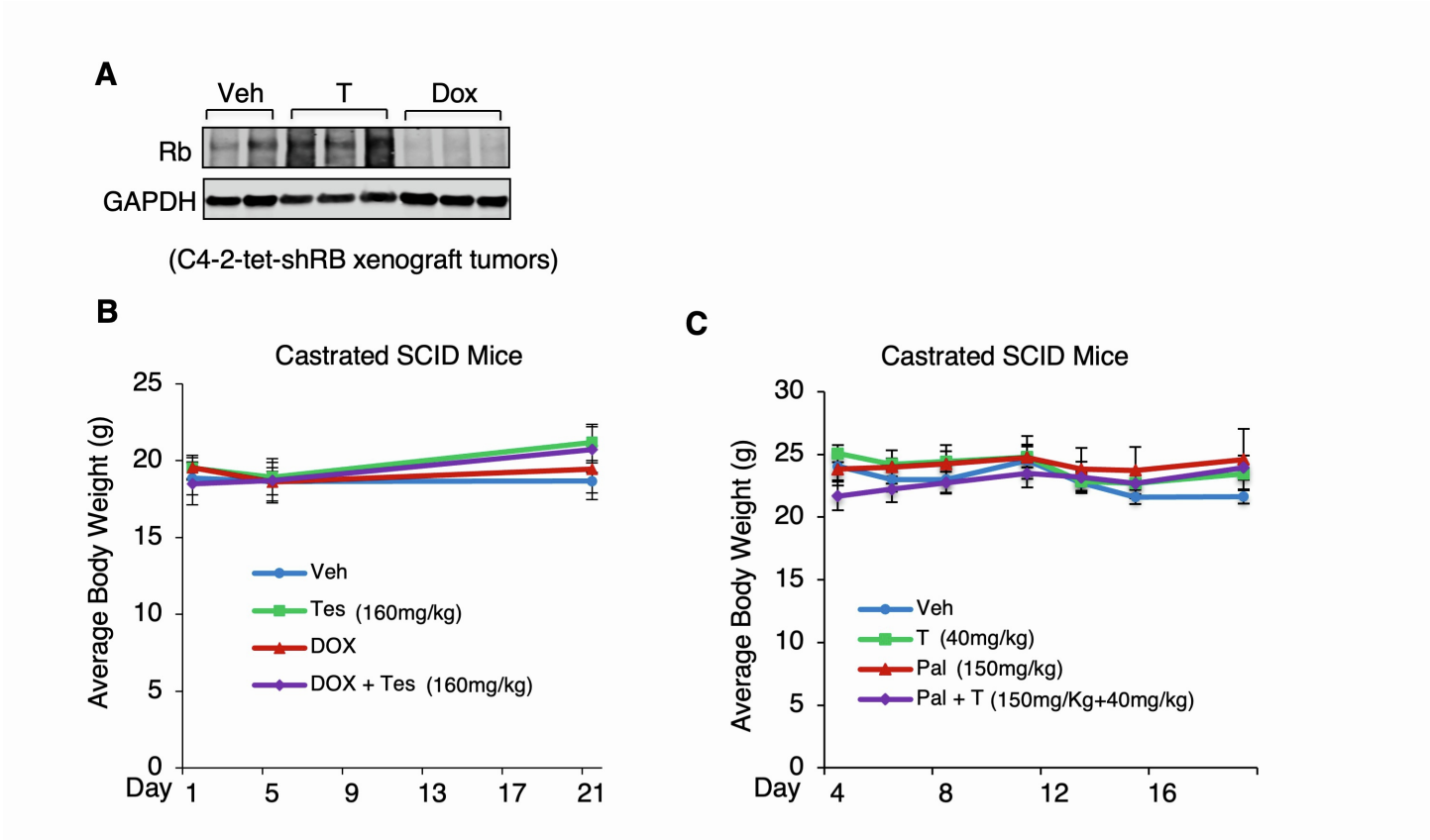


Supplementary Figure S7, related to Figure 3**.**

(**A**) Immunoblotting for Rb and p130 in VCaP cells transfected with siNTC, siRB, siRBL2 (encodes p130), or the combination (3d). (**B**) qRT-PCR for AR-repressed genes in VCaP cells transfected with siNTC, siRB, or siRB+siRBL2 and treated with/out DHT (10nM, 24h).

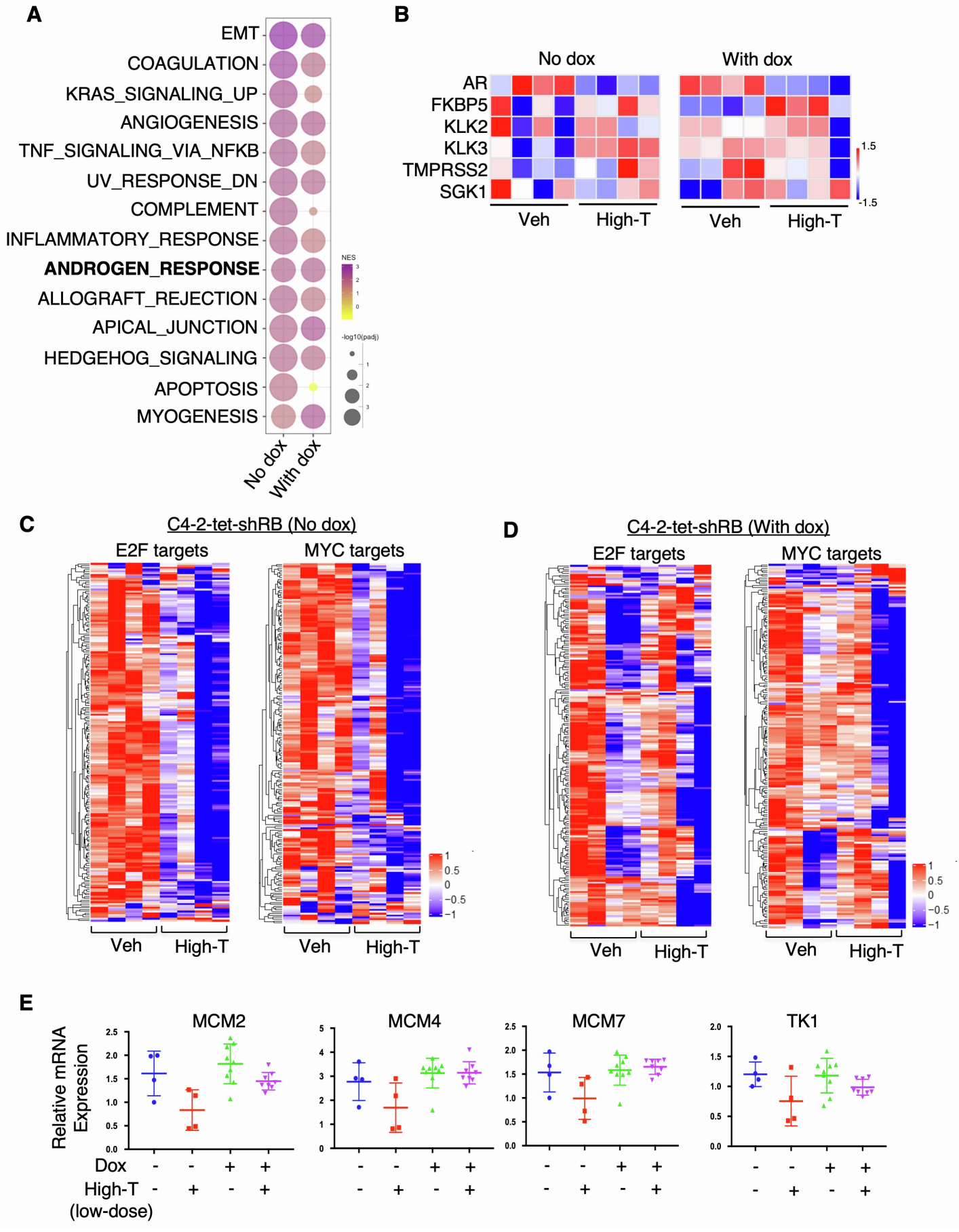
S**upplementary Figure S8**, related to Figure 4.

(**A**) A Venn diagram for AR-activated genes and the gene annotation of the “lost” AR sites. (**B**) Box plots for the expression levels of AR-activated genes containing at least one “lost” AR site in C4-2-tet-shRB cells.



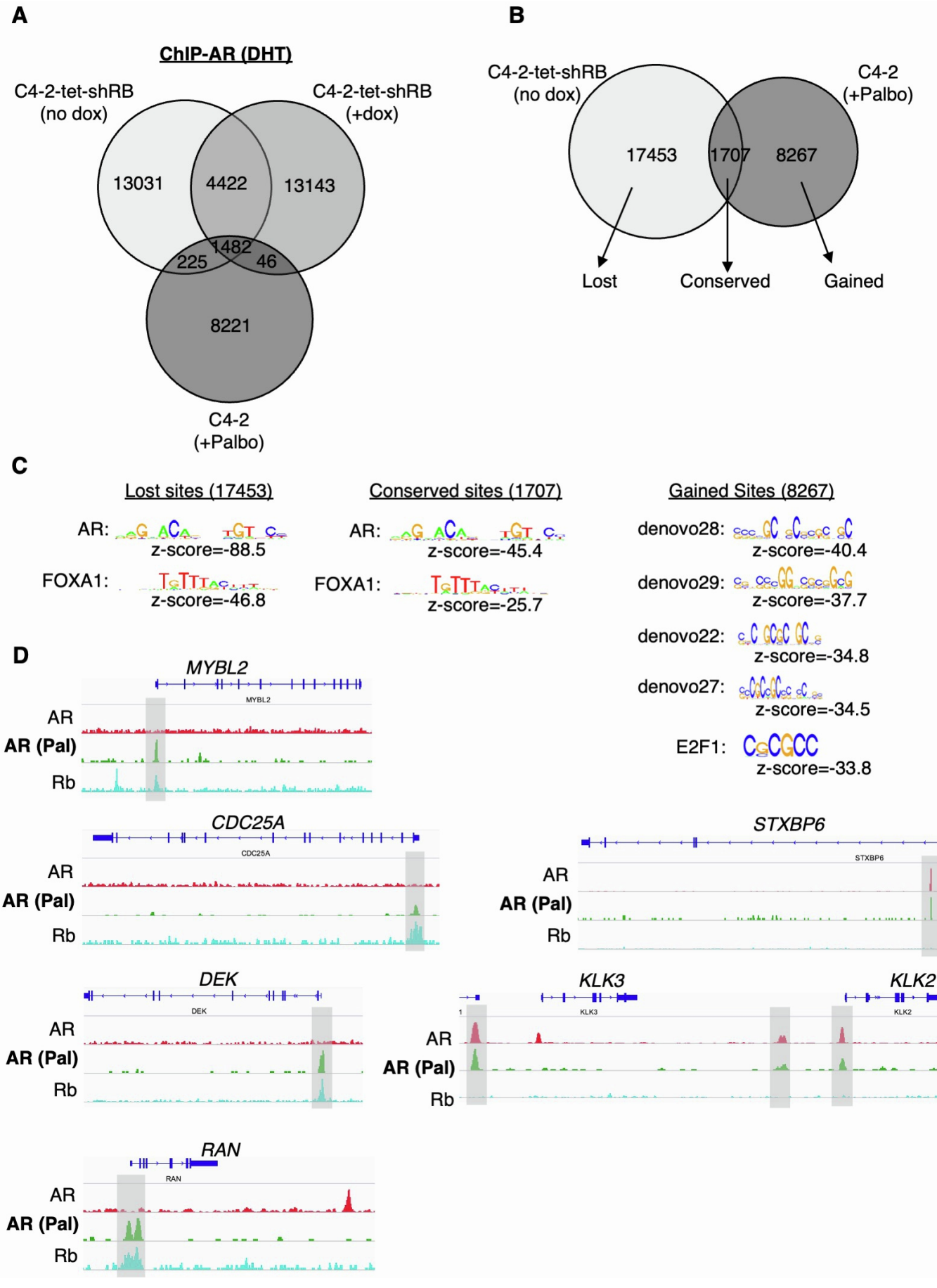
Supplementary Figure S9, related to Figure 5.

(**A**) Immunoblotting for Rb expression in C4-2-tet-shRB xenograft tumor samples from tumor-bearing mice treated with vehicle, 160mg/kg testosterone, and doxycycline supplemented diet. (**B, C**) Bodyweight for castrated male SCID mice receiving daily testosterone (40mg/kg or 160mg/kg) or palbociclib (150mg/kg).


A
EMT
COAGULATION
KRAS_SIGNALING_UP
ANGIOGENESIS
TNF_SIGNALING_VIA_NFKB
UV_RESPONSE_DN
COMPLEMENT
INFLAMMATORY_RESPONSE
ANDROGEN_RESPONSE
ALLOGRAFT_REJECTION
APICAL_JUNCTION
HEDGEHOG_SIGNALING
APOPTOSIS
MYOGENESIS
No dox
With dox
NES
-log10(padj)
B
No dox
With dox
AR
FKBP5
KLK2
KLK3
TMPRSS2
SGK1
Veh
High-T
Veh
High-T
1.5
-1.5
C
C4-2-tet-shRB (No dox)
E2F targets
MYC targets
Veh
High-T
Veh
High-T
D
C4-2-tet-shRB (With dox)
E2F targets
MYC targets
Veh
High-T
Veh
High-T
E
Relative mRNA Expression
MCM2
MCM4
MCM7
TK1
Dox
High-T (low-dose)

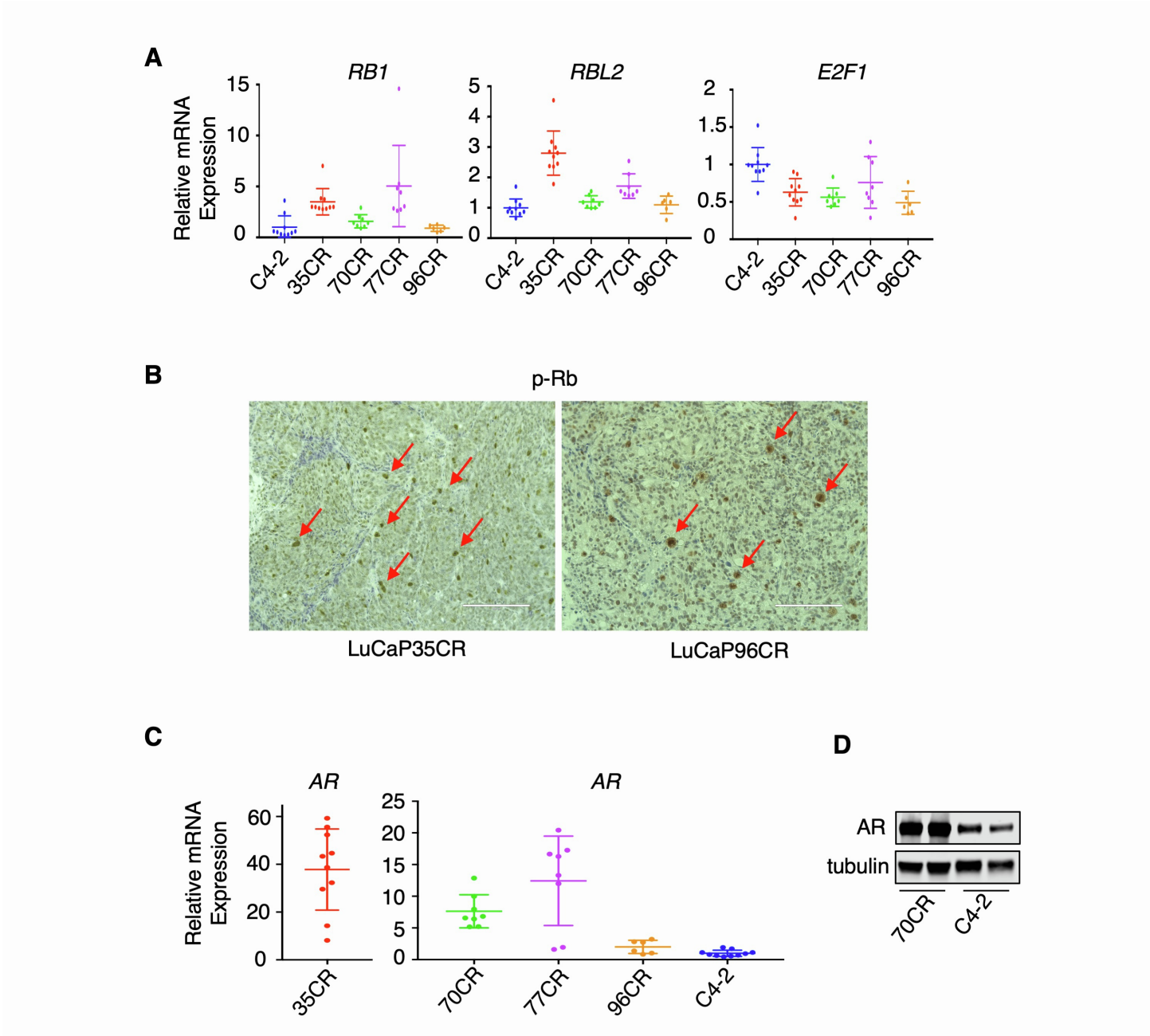
Supplementary Figure S10, related to Figure 6.

(**A**) GSEA was done to compare the androgen-induced genes in Rb-proficient tumors versus Rb-deficient tumors. (**B**) Heatmap view for the expression levels of *AR* and a panel of canonical AR-activated genes in the tumor tissues. (**C**, **D**) Heatmap view for E2Fs target genes and Myc target genes in Rb-proficient (C) versus Rb-deficient tumors (D). (**E**) qRT-PCR for a panel of AR-repressed E2F targets in tumor tissues.


A
ChIP-AR (DHT)
C4-2-tet-shRB (no dox)
C4-2-tet-shRB (+dox)
C4-2 (+Palbo)
13031
4422
13143
1482
225
46
8221
B
C4-2-tet-shRB (no dox)
C4-2 (+Palbo)
17453
1707
8267
Lost
Conserved
Gained
C
Lost sites (17453)
Conserved sites (1707)
Gained Sites (8267)
AR:
z-score=-88.5
FOXA1:
z-score=-46.8
AR:
z-score=-45.4
FOXA1:
z-score=-25.7
denovo28:
z-score=-40.4
denovo29:
z-score=-37.7
denovo22:
z-score=-34.8
denovo27:
z-score=-34.5
E2F1:
z-score=-33.8
D
MYBL2
CDC25A
STXBP6
DEK
KLK3
KLK2
RAN
AR
AR (Pal)
Rb

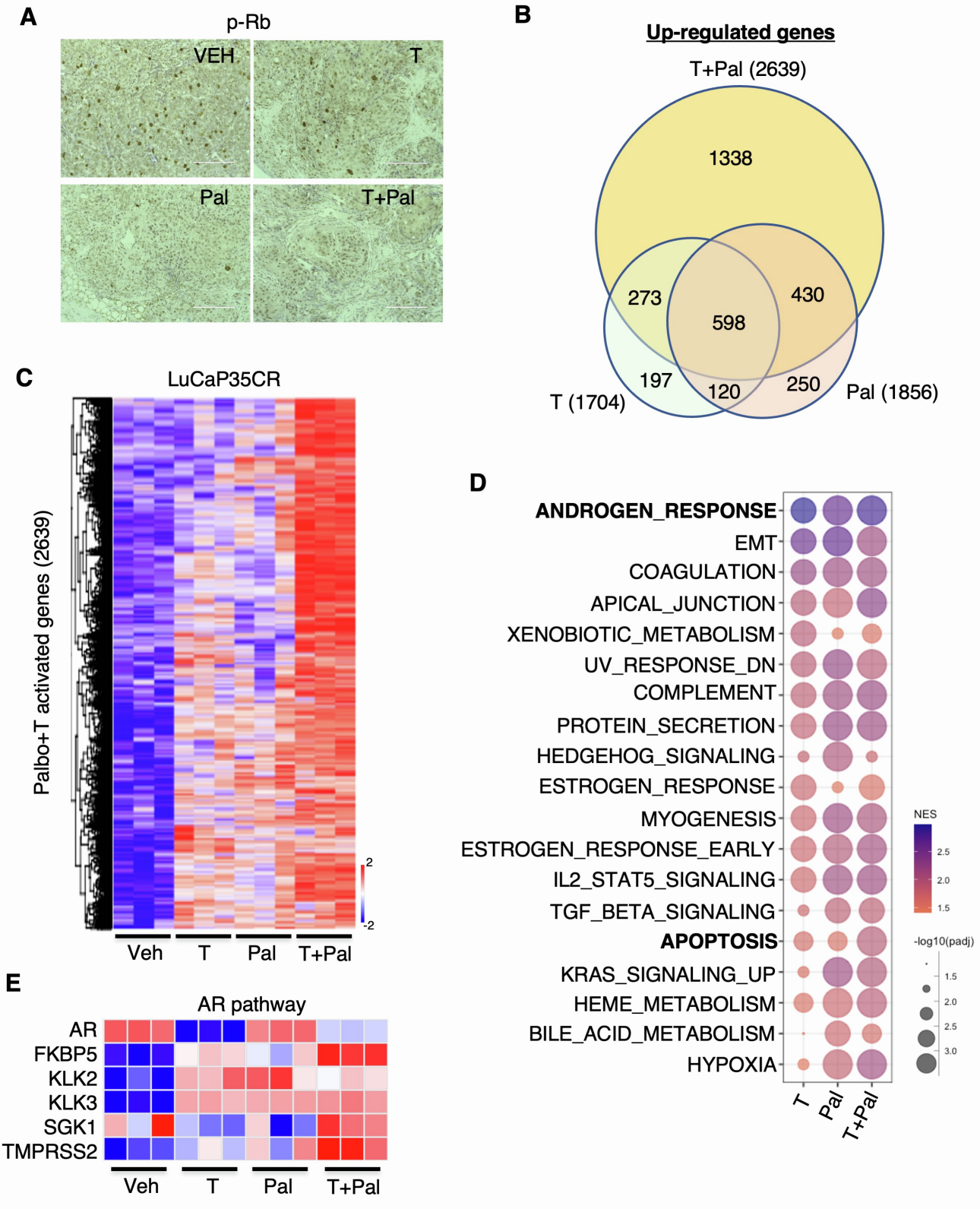
Supplementary Figure S11, related to Figure 7.

(**A**, **B**) ChIP-seq of AR was done in C4-2 cells treated with palbociclib (1μM, 24h) and DHT (10nM, 4h). 9,974 high-confidence peaks were identified using MACS and a Venn diagram was shown to compare AR binding sites under this treatment with AR bindings in C4-2-tet-shRB cells treated with/out doxycycline (3d) and DHT (10nM, 4h). (**B**) “Lost”, “conserved”, and “gained” sites were identified by comparing ChIP-AR peaks under palbociclib+DHT treatment versus DHT only. (**C**) Motif enrichment analysis for these three groups of AR binding sites. (**D**) Examples for gene loci with "gained” AR binding (left) and “conserved” AR binding. Note: STXBP6 is a previously identified AR repressed gene that is not regulated by Rb, and KLK2/KLK3 are classical AR-activated genes.



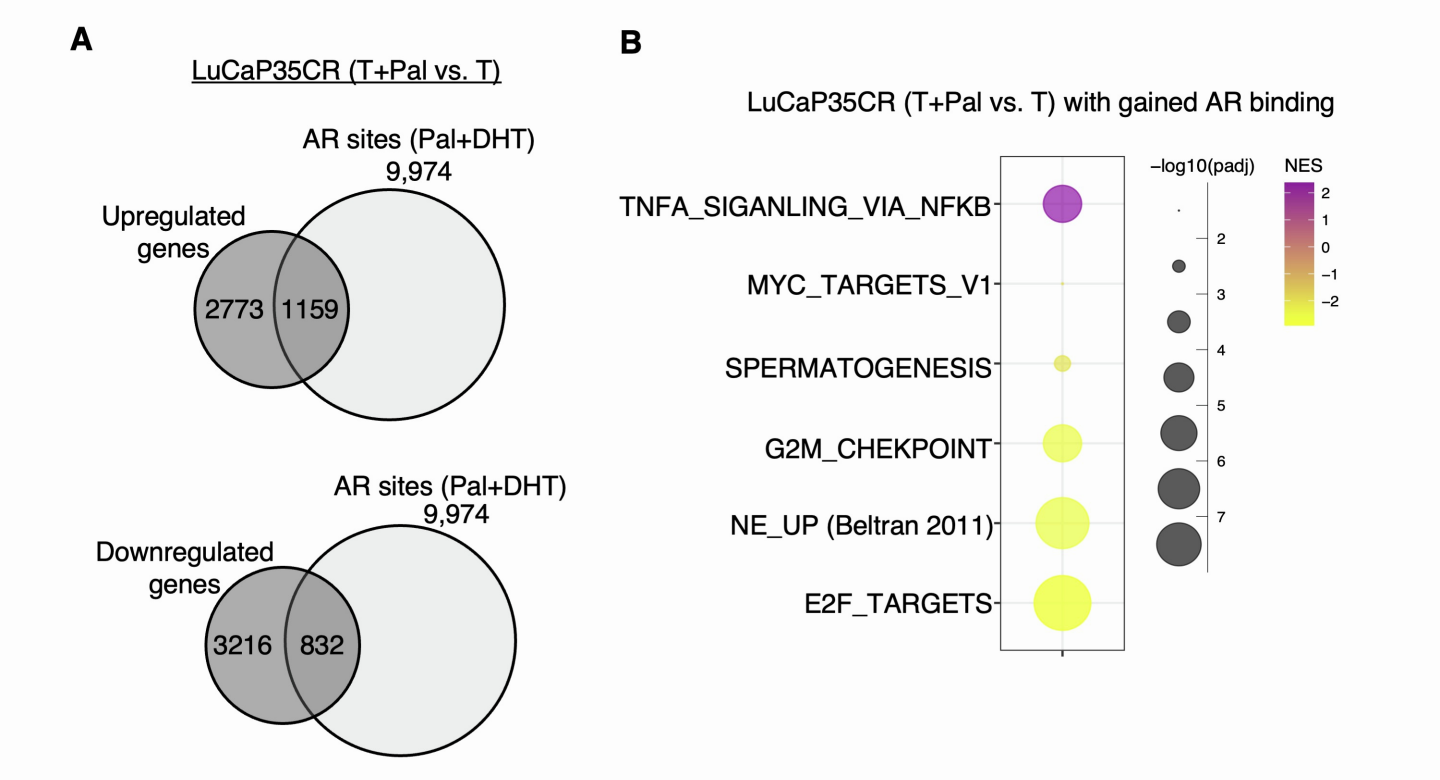
Supplementary Figure S12, related to Figure 7.

(**A**) qRT-PCR for the mRNA expression of indicated genes in tumor samples from C4-2 xenograft and LuCaP PDXs. (**B**) Immunohistochemistry staining of p-Rb (Ser780) in tumor samples from the LuCaP35CR and LuCaP96CR (red arrows indicate cells with high levels of p-Rb). (**C**) qRT-PCR for AR mRNA expression in tumor samples from C4-2 xenograft and LuCaP PDXs. (**D**) Immunoblotting for AR in 70CR and C4-2 xenograft tumor samples.


A
p-Rb
VEH
T
Pal
T+Pal
B
Up-regulated genes
T+Pal (2639)
1338
273
430
598
197
120
250
T (1704)
Pal (1856)
C
LuCaP35CR
Palbo+T activated genes (2639)
2
-2
Veh
T
Pal
T+Pal
D
ANDROGEN_RESPONSE
EMT
COAGULATION
APICAL_JUNCTION
XENOBIOTIC_METABOLISM
UV_RESPONSE_DN
COMPLEMENT
PROTEIN_SECRETION
HEDGEHOG_SIGNALING
ESTROGEN_RESPONSE
MYOGENESIS
ESTROGEN_RESPONSE_EARLY
IL2_STAT5_SIGNALING
TGF_BETA_SIGNALING
APOPTOSIS
KRAS_SIGNALING_UP
HEME_METABOLISM
BILE_ACID_METABOLISM
HYPOXIA
T
Pal
T+Pal
NES
2.5
2.0
1.5
-log10(padj)
1.5
2.0
2.5
3.0
E
AR pathway
AR
FKBP5
KLK2
KLK3
SGK1
TMPRSS2
Veh
T
Pal
T+Pal

Supplementary Figure S13, related to Figure 8.

(**A**) Immunohistochemistry staining of p-Rb (Ser780) in tumor samples from the LuCaP35CR study. (**B**) A Venn diagram for high-T-activated genes, palbociclib-activated genes, and the combination treatment-activated genes. (**C**) Heatmap view for the combination treatment-activated genes in all tumor samples. (**D**) GSEA for the upregulated genes by single treatments versus the combination treatment. (**E**) Heatmap view for the expression levels of *AR* and a panel of canonical AR-activated genes in the tumor tissues.



Supplementary Figure S14, related to Figure 8.

(**A**) Identification of up/down-regulated genes (fold-change>1.5) by the combination treatment versus testosterone alone and associated with nearby AR binding sites determined in C4-2 cells treated with DHT+palbociclib. (**B**) GSEA for these up/down-regulated genes.

Supplementary Table S1, related to Figure 2

E2F target gene (200) expression (RPKM) in C4-2-tet-shRB cells. Note: DHT-downregulated genes (fold-change < 1.5) are highlighted in red.

SUPPLEMENTARY METHODS

ChIP-seq analysis

The preliminary sequencing output is supplied in FASTQ format. FastQC (version 0.11.9) was then used to check sequence quality. ChIP-sequencing reads were mapped to the hg19 human genome using bwa (version 0.7.9a) with aln and samse sub-commands. When processing bwa aln, the first 32 subsequences were taken as seed and the parameter for reads trimming is 5 (-l 32 -q 5). Samtools (version 1.2) was used to convert sam files to bam format. The significance of enriched ChIP regions was evaluated by using MACS2 (version 2.1.0)[7] with fix-bimodal turned on and extend size set as 100 (--bw 250 –mfold 10 30 –fix-bimodal –extsize 100). bedGraph files generated from MACS2 were converted into bigwig files with UCSC tools (version 369) as follows: bedGraph files were sorted by bedSort, then clipped with bedClip and converted to bigwig format using bedGraphToBigWig. The import.bed function from R package rtracklayer (version 1.44.4) and the findOverlaps function from R package IRanges (version 2.18.3) were used to analyze peak intervals and determine the overlapped regions. All Venn diagrams were generated using VennDiagram (version 1.6.20) R package.[8] The signals associated with genomic regions were visualized by using compueMatrix and plotHeatmap tools from deepTools (version 3.3.0).[9] computeMatrix with reference-point mode was used to calculate scores for each genomic region, and plotHeatmap was used to create a heatmap for scores associated with genomic regions. Motif enrichment analysis was performed by using SeqPos with the default setting in Galaxy/Cistrome.[10] The top 5000 peaks were determined by ranking $-\log_{10}$(p-value) from MACS results. Binding and Expression Target Analysis (BETA) was performed by BETA software package (version 1.0.7)[11] with default parameters to integrate ChIP-seq with differential gene expression to predict direct targets. Peak interval files from MACS2 and differential expression results from limma were used as inputs.

RNA-seq analysis

Transcriptome-sequencing reads were aligned to the human reference genome (hg19) using STAR (version 2.7.2) followed by counting with featureCounts (version 1.6.4) from GRCh37 Ensembl reference. Genes that were not expressed at a level greater than or equal to 1 count per million reads were excluded from further

analysis. All gene counts were processed with R package limma (3.40.6)[12] to evaluate the differential expression using the Benjamini–Hochberg false discovery rate (FDR)-adjusted *P*-value. The expression values were centered and scaled across samples and then displayed using ComplexHeatmap (version 2.0.0) R package. Gene Set Enrichment Analysis (GSEA) was conducted using pre-ranked gene lists by R package fgsea (version 1.10.1). The top pathways (*P*-value<0.05) ranked by normalized enrichment scores (NES) were plotted for visualization. Gene sets were considered significantly enriched with FDR *q*-value<0.25 and *p*-value<0.05. For identification of AR regulated genes, AR-upregulated and AR-downregulated genes were determined by fold-change>2 and *P*-value<0.05 in the comparison of C4-2-tet-shRB cells (no doxycycline) with DHT treatment versus those with ethanal treatment and with nearby AR peaks found in control C4-2 cells in the presence of DHT. The steps to generate noncanonical AR-regulated genes are: (i) genes were selected by fold-change>1.5 and *P*-value<0.05 in the comparison of short-term *RB1*-silenced cells with DHT versus ethanal; (ii) these genes were further filtered based on the condition that the fold-change of AR-regulated gene expression after *RB1* silencing versus control is great than 2; (iii) the final gene set was generated by examining whether the gene contains “gained” AR binding in C4-2-tet-shRB cells (with doxycycline) in the presence of DHT.

ChIP-qPCR

For preparation of ChIP, cells were fixed with 1% formaldehyde and then lysed by the ChIP lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris–HCl pH 8.1). Chromatin was then sheared to 500-800 bp fragments using Bioruptor Sonicator (Diagenode). Immunoprecipitation was carried out using anti-p130 antibody (Cell Signaling). DNA fragments were purified and analyzed by qPCR using SYBR Green (Thermo Fisher Scientific) with primer sets of MCM-pro, TK1-pro, LMNB1-pro, FANCI-pro, BLM-enh, KLK3-enh, NKX3.1-enh (listed previously).[2]

Quantitative RT-PCR

RNA from the cell line was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instruction. For RNA from tumor tissue samples, ~30 mg of tumor tissue was homogenized by TissueLyser LT (Qiagen) with one 5 mm bead, and then followed by using RNeasy Kit (Qiagen) to isolate total RNAs. 20 ng of total RNA was

used for quantitative real-time PCR (qRT-PCR) with Fast 1-step Mix (Thermo Fisher Scientific) using QuantStudio 3. All qRT-PCR data were normalized with an internal control GAPDH and quantitated using ΔΔCt method. Taqman primers and probes used in this study were all predesigned by and obtained from Thermo Fisher Scientific.

Immunohistochemistry staining

Immunohistochemistry (IHC) staining was performed on formalin-fixed paraffin-embedded tumor tissue sections using anti-p-Rb (Ser780) antibody (Cell Signaling). IHC was performed by iHisto.

SUPPLEMENTARY REFERENCES

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