SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Tumor tissue arrays and statistical analysis

The prostate tissue microarrays used in this study were prepared by NYU Cooperative Prostate Cancer Tissue Resource as described previously (3). Briefly, three intermediate-density prostate tissue arrays consisted of a total 429 cases including 18 hormone resistant (HR) transurethral resection (TURP) specimens of prostate from patients with clinically advanced prostate cancer, 371 cases of HN prostate cancer tissue (Gleason Sum 6-10) from the radical prostatectomy specimens of patients with clinically localized prostate cancer, and 40 cases of non-neoplastic containing tissue from patients with benign prostatic hypertrophy. The determination of HN and HR was described previously (3). All cases upon collection into the resource (under an IRB approved protocol) had repeat pathology characterization of tissues and review of medical records.

The pair-wise group comparison was conducted by non-parametric Kruskal-Wallis test. Kaplan-Meier analysis was used for testing the association of AR3 staining and PCA recurrence. The statistical analyses were carried out by using SAS version 9.1 software (SAS Institute Inc. Cary, NC, USA).

Microarray Analysis

Total RNA was extracted from two biological replicates of CWR-R1 and 22Rv1 cells treated with shAR3-1, shARa and the scrambled shRNA control, respectively. Hybridization was carried out using the conditions specified in the Agilent Human Whole Genome Expression system. Scanned images were processed for quality assessment and preprocessing of image data using the Agilent Feature Extraction software (Agilent Technologies). The software quantifies feature signals and their background, performs

dye normalization and calculates feature log ratios and error estimates. The error estimates, based on an extensive error model and pixel level statistics calculated from the feature and background for each spot, are used to generated a p-value for each log ratio. The differential expressed genes were identified by their p-value < 0.05 and a minimal 1.4 fold change in both cell lines treated with a given specific shRNA compared to the scrambled control. The changes of some identified genes were validated by the quantative real-time PCR. The raw data of the microarray analysis have been deposited in the GEO database (accession # GSE13919).

Supplementary figure 1:

Human AR3 full-length cDNA sequence

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GACACTGAATTTGGAAGGTGGAGGATTTTGTTTTTTTCTTTTAAGATCTGGGCATCTTTT
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CGCTATGGGGACCTGGCGAGCCTGCATGGCGCGGGTGCAGCGGGACCCGGTTCTGGGTCA
 R  Y  G  D  L  A  S  L  H  G  A  G  A  A  G  P  G  S  G  S
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G G G E A G A V A P Y G Y T R P P Q G L
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N C P S C R L R K C Y E A G M T L G **E K**
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CCTTAATAAAGCCATTAATACACCCAAAAAAAAAAAAAAAA

Supplementary Figure 1, 2 and 3. Nucleotide and deduced amino acid sequence of human AR isoforms. The stop codons upstream of and in-frame with the first putative methionine is in red and *underlined*. The termination codon is indicated by "-", and the polyadenylation signal is in red and *underlined*. The AR isoform unique amino acids are in bold and *underlined*.

Supplementary figure 2:

Putative human AR4 full-length cDNA sequence

```
GACACTGAATTTGGAAGGTGGAGGATTTTGTTTTTTTCTTTTAAGATCTGGGCATCTTTT
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                                  M  E  V  Q  L  G  L  G  R  V  Y
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 E  V  I  Q  N  P  G  P  R  H  P  E  A  A  S  A  A  P  P  G
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Supplementary figure 3:

Putative human AR5 full-length cDNA sequence

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Supplementary figure 4:

Putative human AR6 full-length cDNA sequence

```
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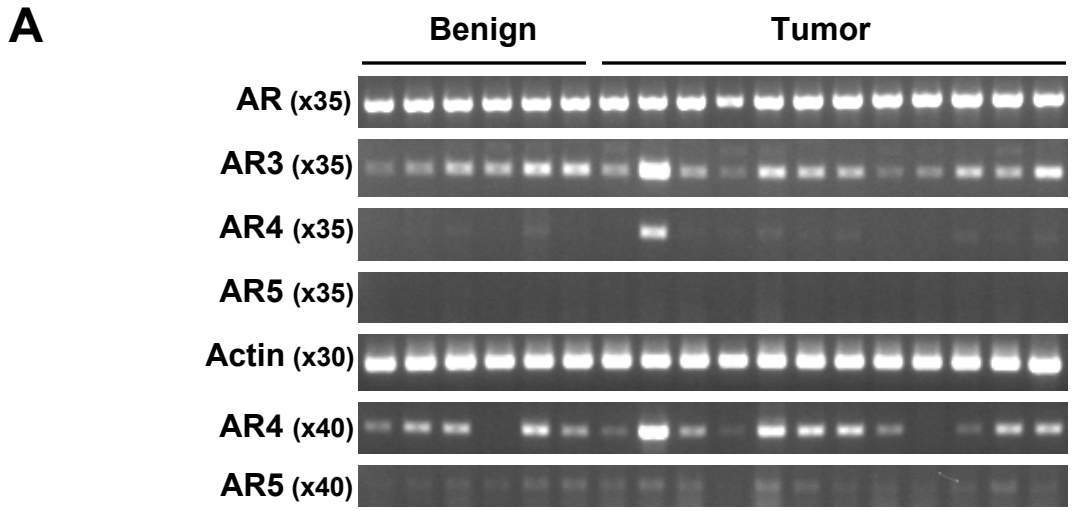
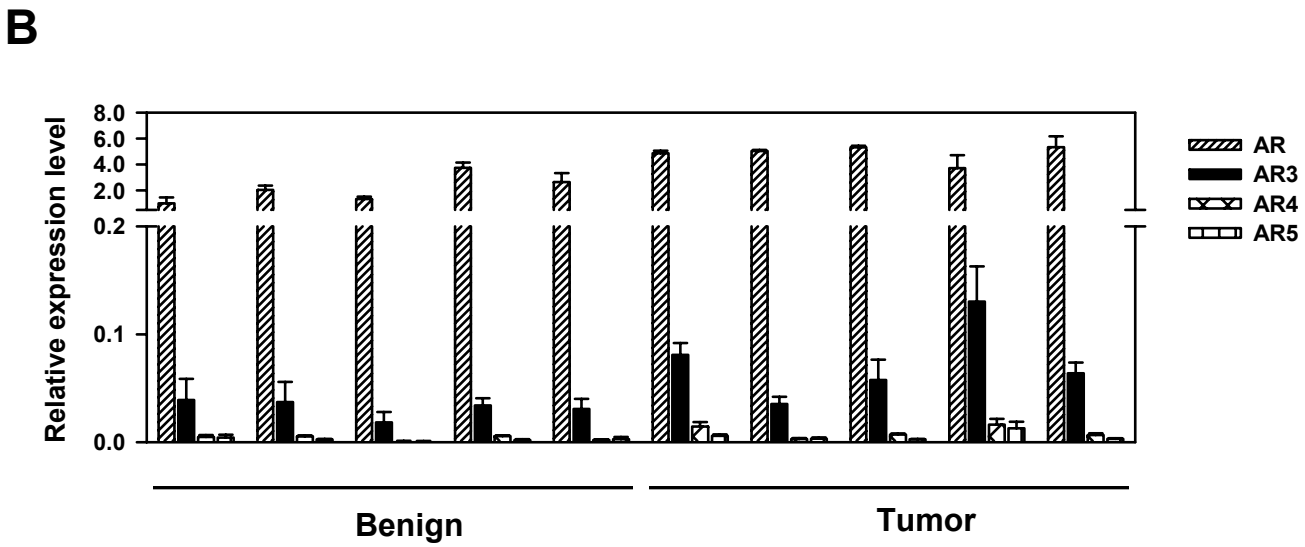
Supplementary figure 5



Expression of AR, AR3, AR4 and AR5 in human prostate tissues. Total RNA was isolated from 6 benign and 12 malignant human prostate tissues and subjected to reverse transcription-PCR. The primer sets used to amplify the AR, AR3, AR4 and AR5 isoform specific transcripts were described in methods. 30-40 cycles of amplification were used as indicated in brackets.



Expression of AR, AR3, AR4 and AR5 in human prostate tissues. Total RNA was isolated from 5 benign and 5 malignant human prostate tissues and subjected to reverse transcription-PCR. The primer sets used to amplify the AR, AR3, AR4 and AR5 isoform specific transcripts were described in methods.

Supplementary figure 6

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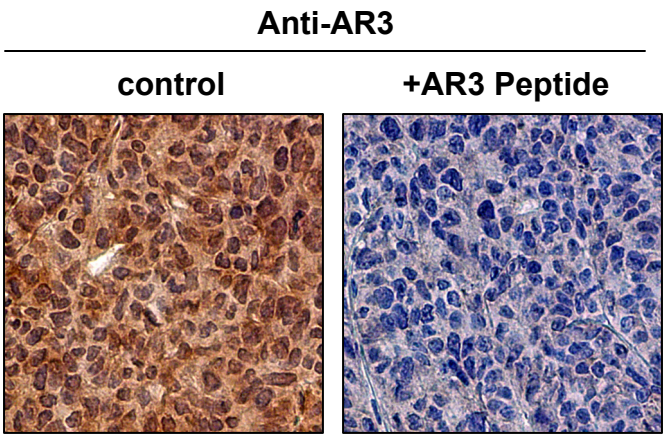

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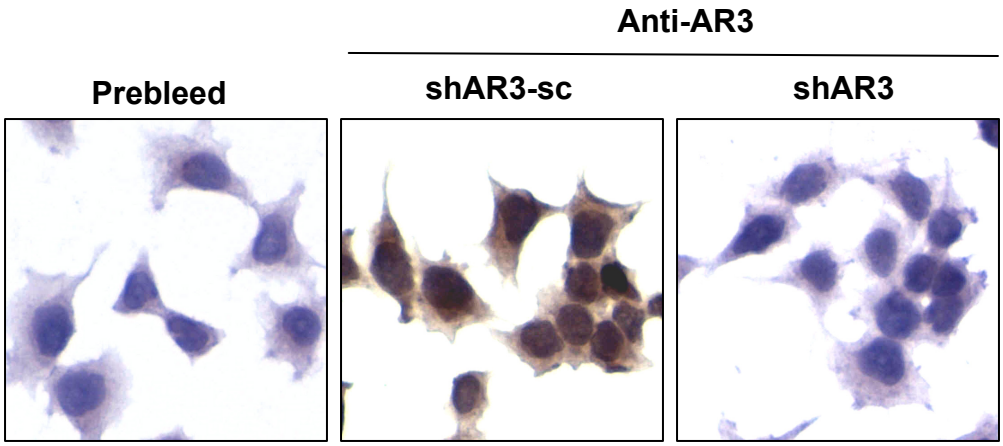
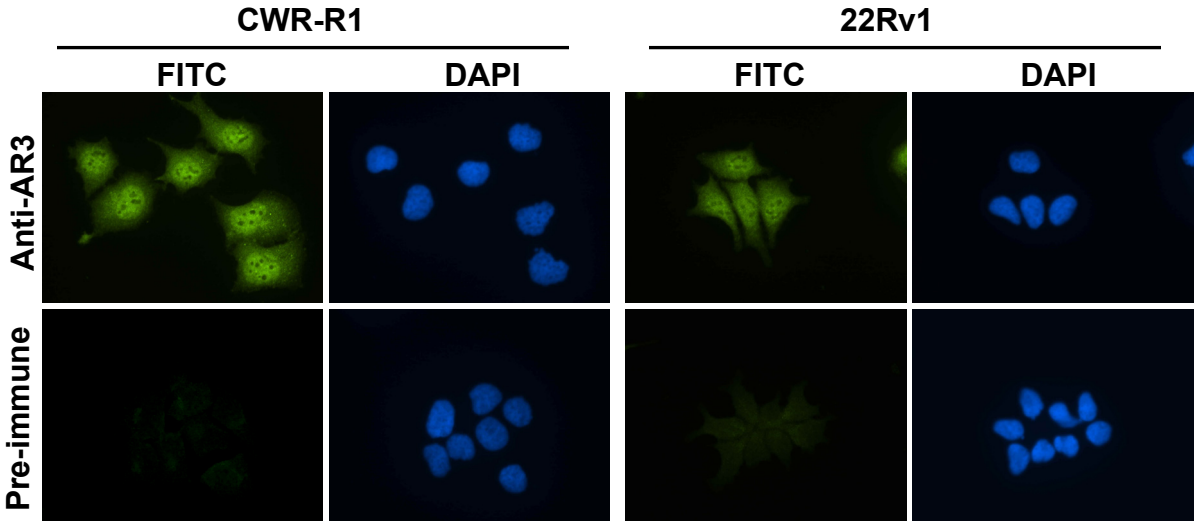


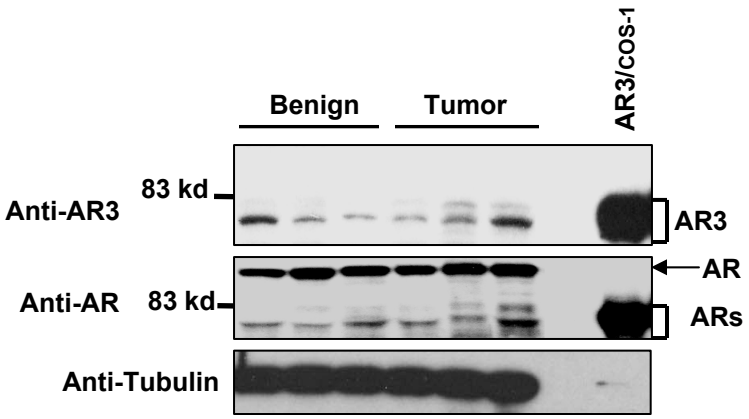
Specificity of the anti-AR3 antibody. (**a**) Prostate tissues were immunostainned with anti-AR3 antibody, with or without addition of the AR3 peptides. (**b**) CWR-R1 cells were infected with the lentivirus encoding AR3 shRNA (shAR3) and the scrambled control AR3 shRNA (shAR3-sc). At 48 hr post infection, the cells were subjected to immunocytochemistry analysis. The same antigen retrieval procedure was used as in IHC staining of human tissues described in the Methods.

Supplementary figure 7



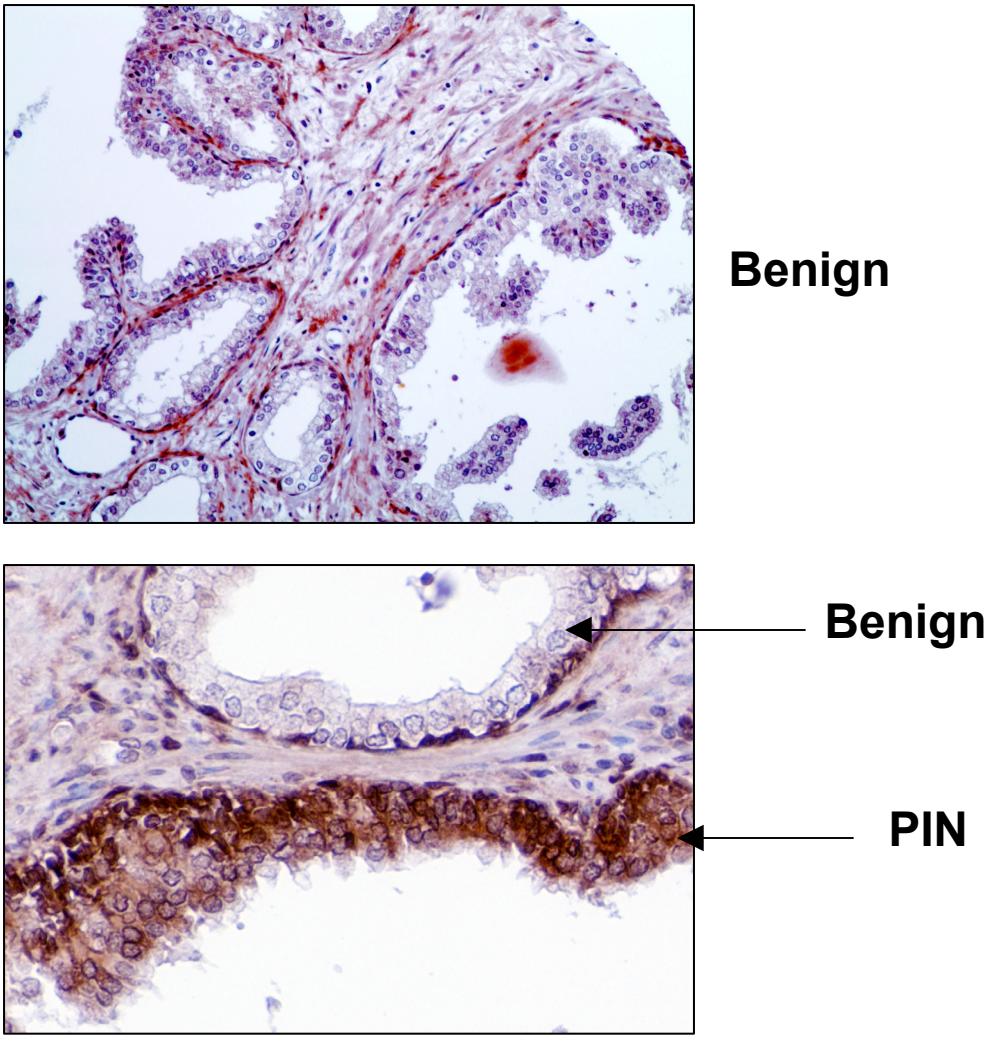
Subcellular Localization of AR3. CWR-R1 and 22Rv1 cells were subjected to immunofluorescence staining with anti-AR3 antibody or the control pre-immune serum. The nucleus was visualized with DAPI staining.

Supplementary figure 8



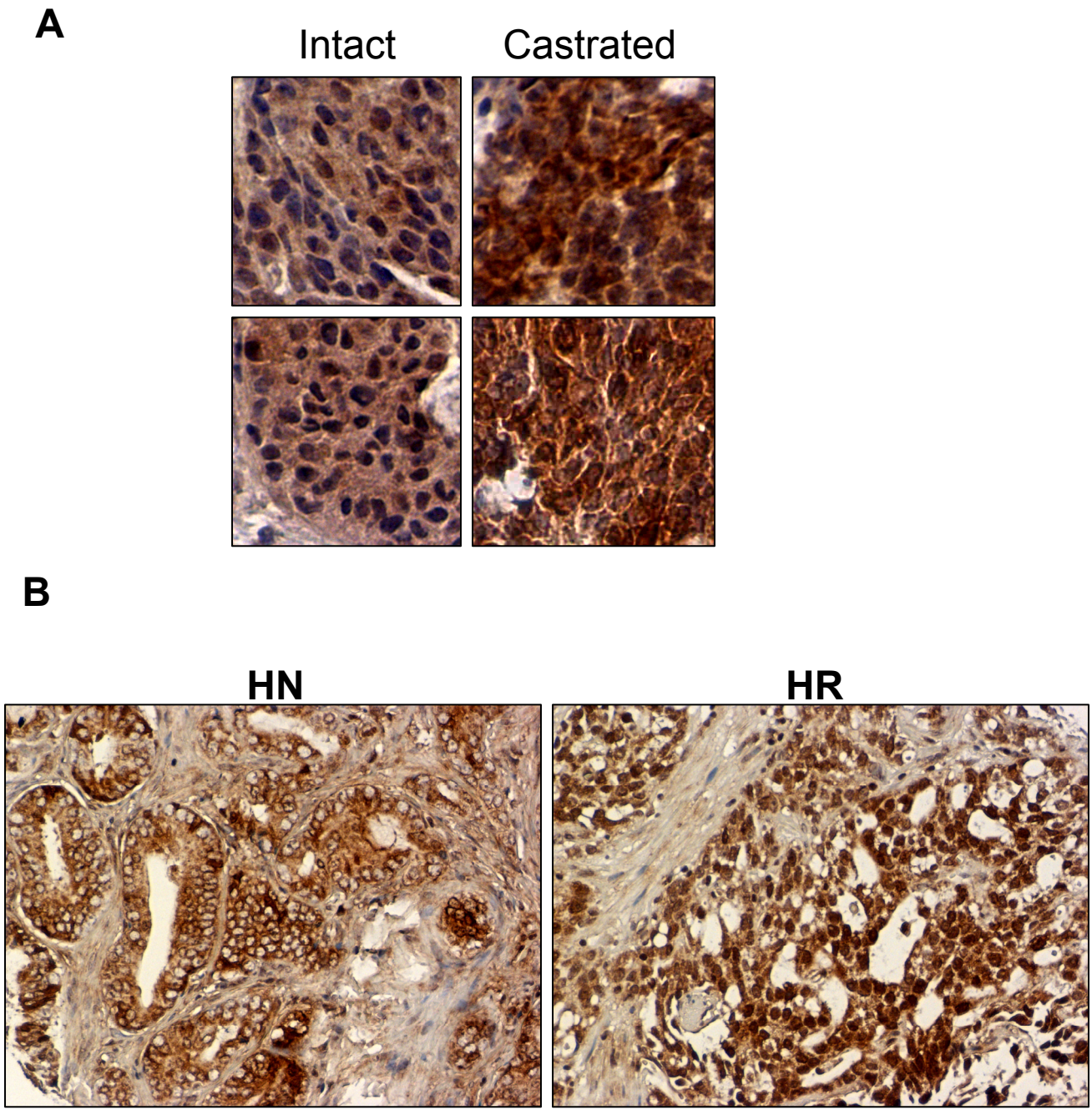
Expression of AR3 in human prostate tissues.
The total protein extracts from human benign and cancerous prostate tissues (Gleason Grade ranged 6-7) were subjected to Western Blot with anti-AR3 and anti-AR, respectively. Overexpressed AR3 in COS-1 cells was used as a positive control. Tubulin was used as a loading control.

Supplementary figure 9



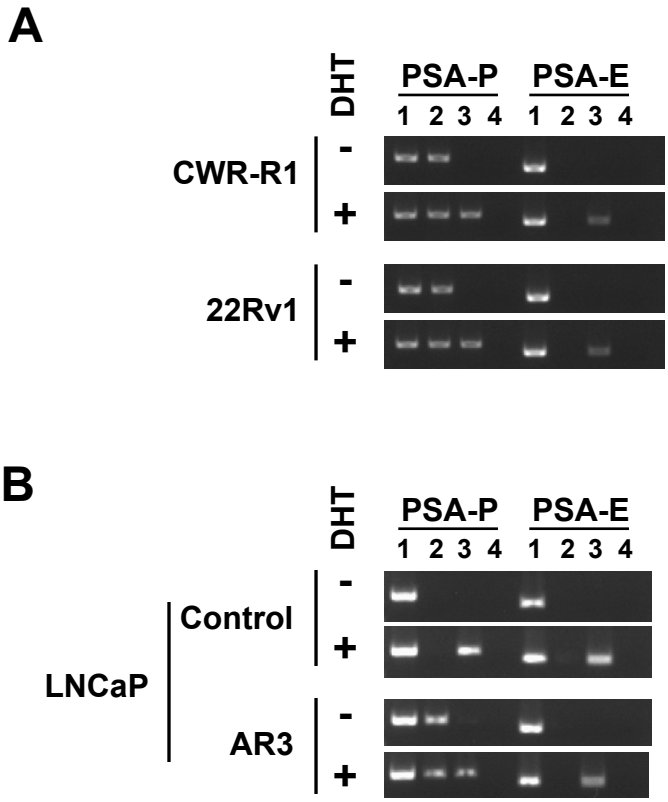
IHC staining of AR3 in human prostate tissues shows that very little staining in benign luminal epithelial cells but positive staining in basal cells and stroma. However, AR3 is detectable in the adjacent PIN lesion.

Supplementary figure 10



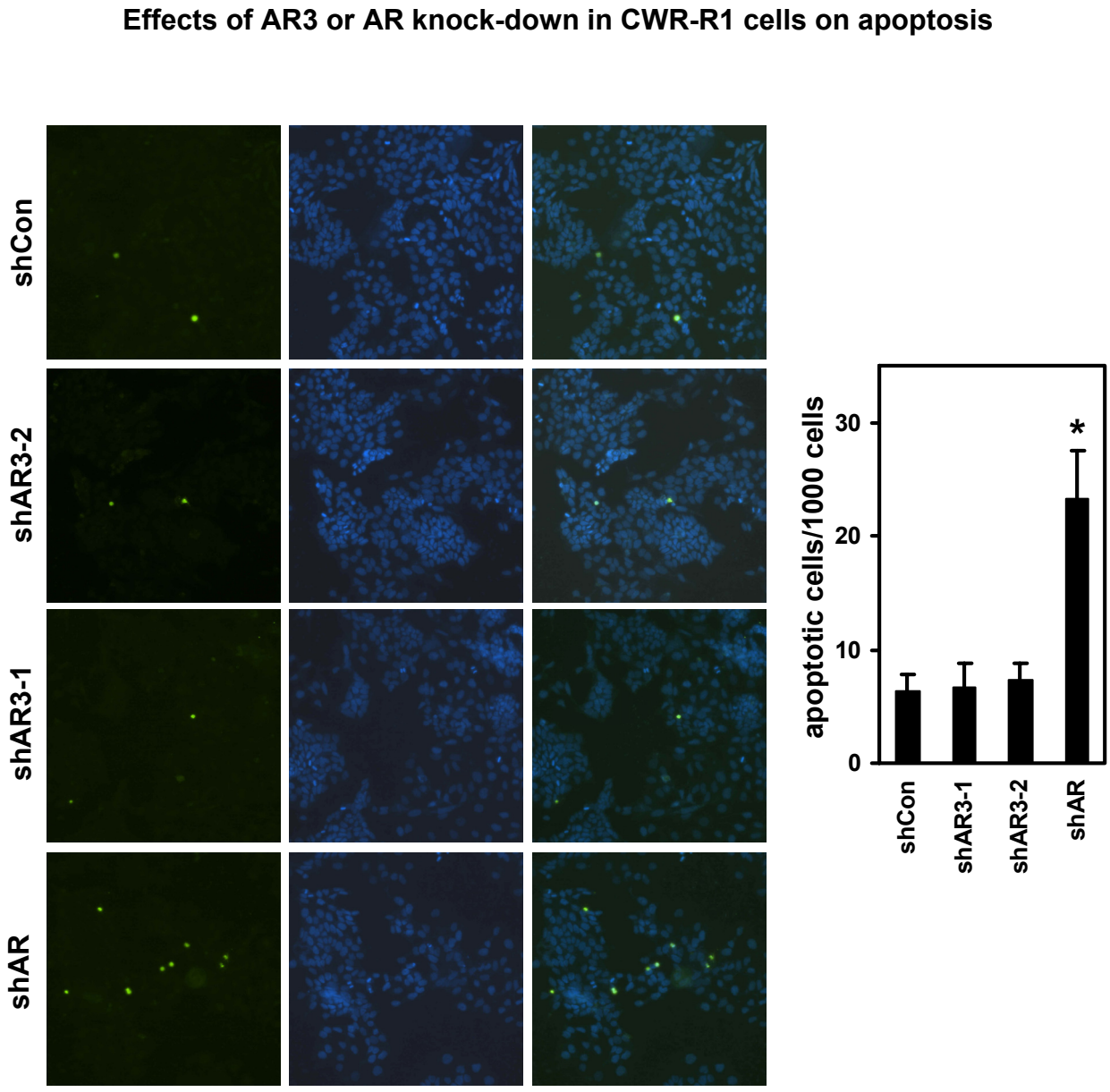
IHC staining of AR3 on two pairs of matched CWR22 xenografts derived from the intact or castrated mice (**A**) and a pair of matched human prostate tumor samples from the same patient (**B**).

Supplementary Figure 11



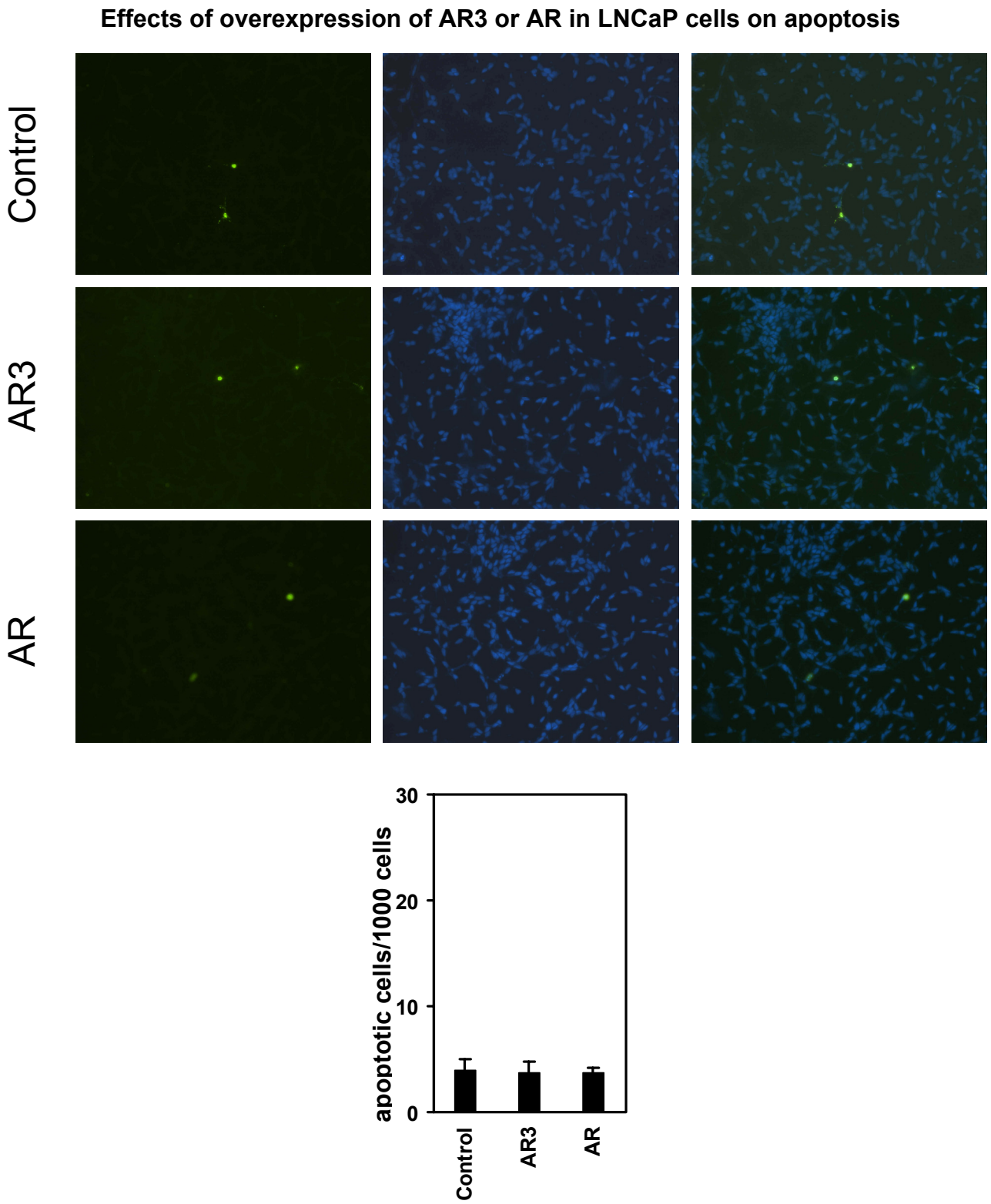
Recruitment of AR3 to the promoter region but not the enhancer region of PSA gene. (**A**) CWR-R1 and 22Rv1 cells were treated with DHT (10 nM) for 1 hr. Binding of AR3 or AR to the promoter ARE site (PSA-P) and to the enhancer ARE site (PSA-E) of human *PSA* gene was analyzed by the ChIP assay. PCR products from input (1), immunoprecipitation with anti-AR3 antibody (2), anti-AR antibody (3) or the control antibody (4), were resolved on agarose gels. (**B**) LNCaP cells were transfected with AR3 expressing vector or vector control. 24 hr posttransfection, the cells were treated with or without DHT (10 nM) for 1 hr and subjected to ChIP as in (**A**).

Supplementary Figure 12



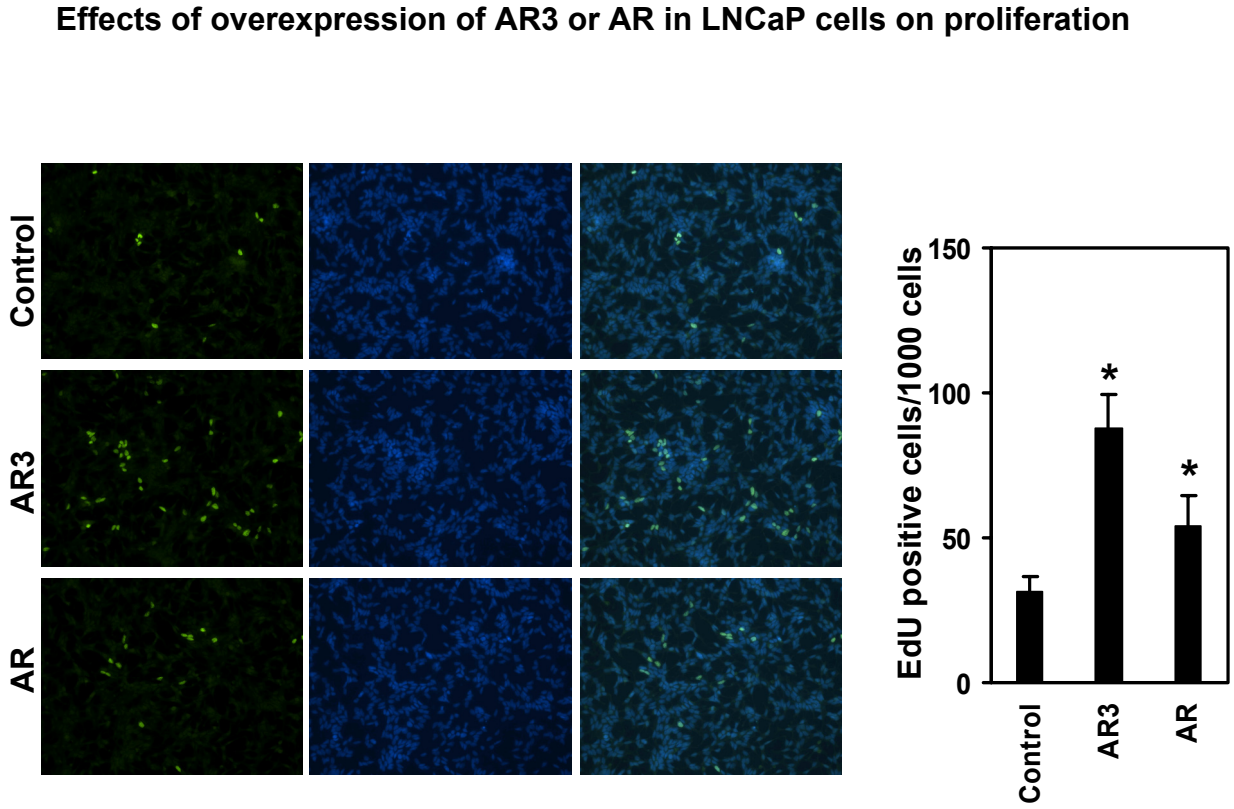
CWR-R1 cells were infected with lenti-virus encoding specific shRNA for AR3 (shAR3-1 or shAR3-2) or AR (shAR) or the scrambled control shRNA (shCon) in androgen-depleted medium. At 48 h post-infection, the apoptosis was detected by TUNEL assays. Apoptotic cells were quantified by counting TUNEL-positive cells in 1000 cells from three random independent fields. *, $p < 0.05$ compared with the control.

Supplementary Figure 13



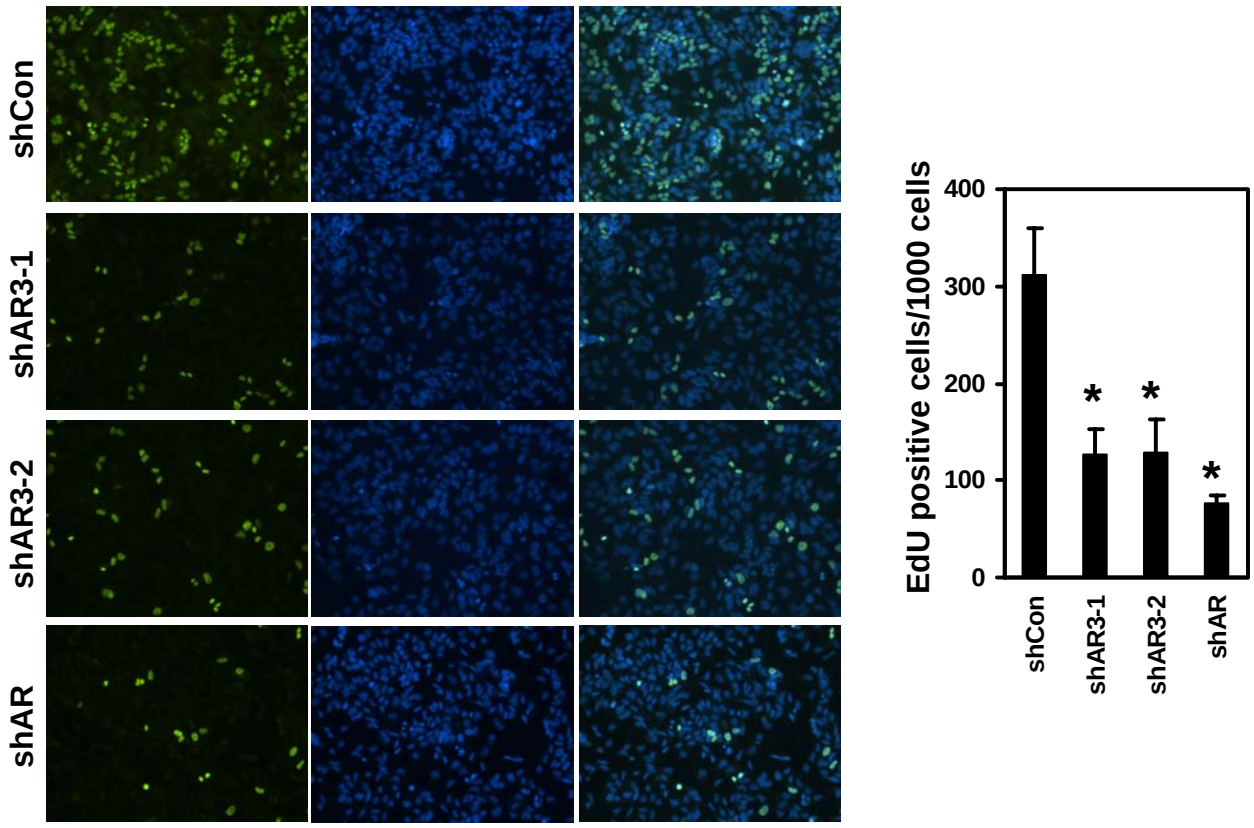
LNCaP cells were plated on poly-L-lysine-coated coverslips and infected with the lentivirus encoding the AR3 or AR expression vector or the control vector in androgen-depleted medium. At 48 h post-infection, apoptotic cells were quantified by counting TUNEL-positive cells in 1000 cells from three random independent fields.

Supplementary Figure 14A



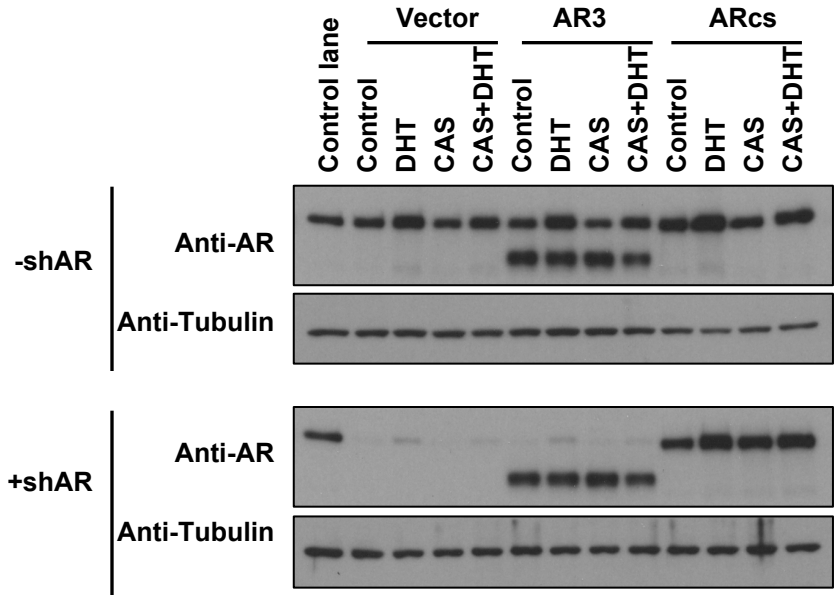
LNCaP cells were plated on poly-L-lysine-coated coverslips and infected with the *lenti*-virus encoding the AR3 or AR expression vector or empty vector (Control) in androgen-depleted medium. At 48 h post-infection, the cell proliferation was evaluated by the Click-iT™ EdU Assay (Invitrogen). Cell proliferation was quantified by counting the number of EdU-positive cells in 1000 cells from three random independent fields. *, $p < 0.05$ compared with the control.
EdU: an improved Thymidine analogue.

Supplementary Figure 14B



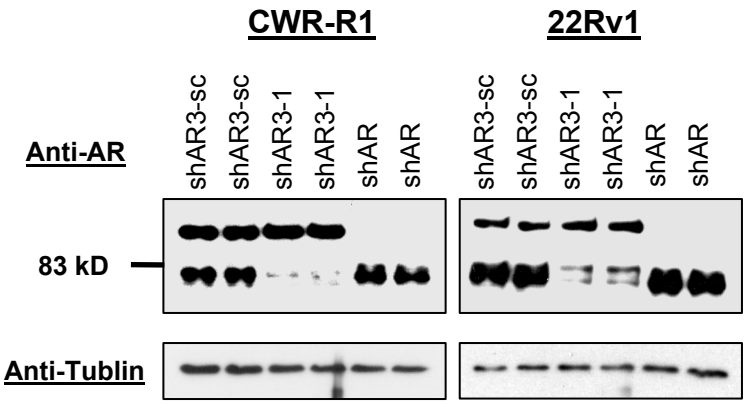
CWR-R1 cells were plated on coverslips and infected with lenti-virus encoding two specific shRNAs against AR3 (shAR3-1, shAR3-2) or AR (shAR) or scrambled control shRNA (shCon) in androgen-depleted medium. Cell proliferation was evaluated by the Click-iT™ EdU Assay as in A. *, $p < 0.05$ compared with the shCon.

Supplementary Figure 15



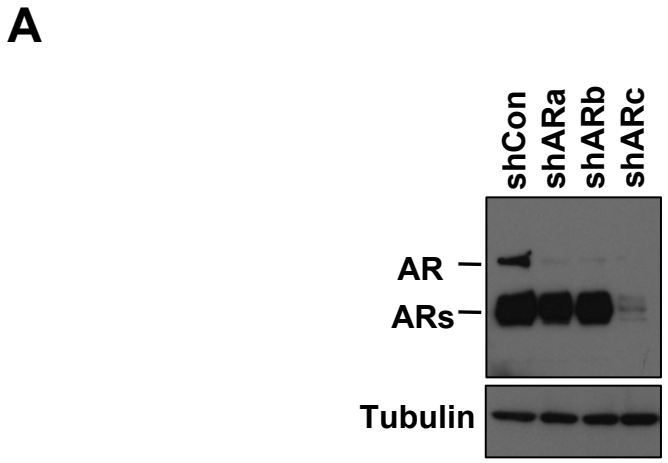
The expression level of AR and AR3 in the cell lysates used for luciferase assays described in Fig. 2D was determined by western blot with anti-AR. The first lane of each blot is served as the reference loading control for all blots. the upper panels (-shAR) were samples without shAR treatment and the lower panels (+shAR) were treated with the shAR.

Supplementary Figure 16

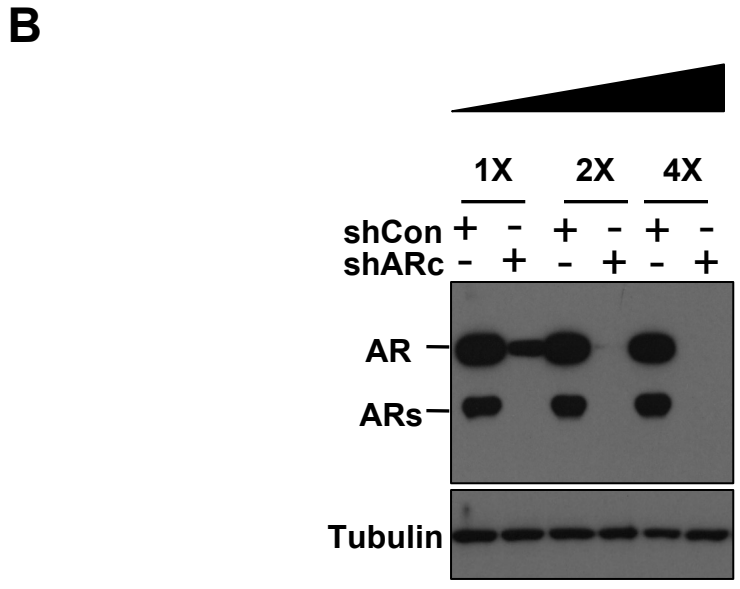


Expression of AR proteins in CWR-R1 and 22Rv1 cells treated with AR3/AR shRNAs used in Microarray experiments. CWR-R1 and 22Rv1 cells were infected with *lenti*-virus encoding specific shRNA against AR3 (shAR3-1) or AR (shAR) or the scrambled control shRNA (shAR3-sc). At 48 hr post-infection, the cell lysates were blotted with anti-AR and anti-Tubulin antibodies, respectively. The cells treated in parallel were subjected to RNA purification and microarray analysis.

Supplementary Figure 17



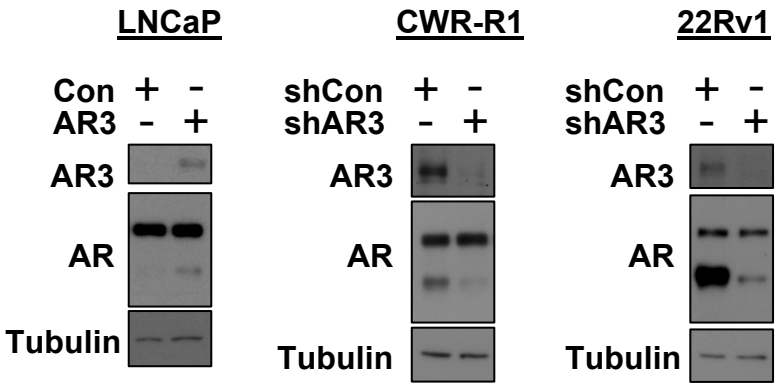
AR shRNAs Selectively knockdown the AR long/short forms in 22Rv1 cells. 22Rv1 cells were infected with *lenti*-virus encoding AR shRNAs (shARa, shARb, shARc) targeting different regions of AR as indicated in (**Fig.1D**), as well as control shRNA (shCon), at 48 h post-infection, total cell lysates were subjected to western-blot with anti-AR and anti-Tubulin antibodies, respectively.



Effects of shARc on expression of the full-length AR and the truncated form AR (ARs). CWR-R1 cells were infected with the increasing doses of *lenti*-virus encoding AR shRNAc (shARc) and control shRNA (shCon). At 48 h post-infection, total cell lysates were subjected to western-blot with anti-AR and anti-Tubulin antibodies, respectively.

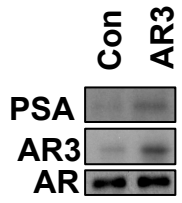
Supplementary Figure 18

A



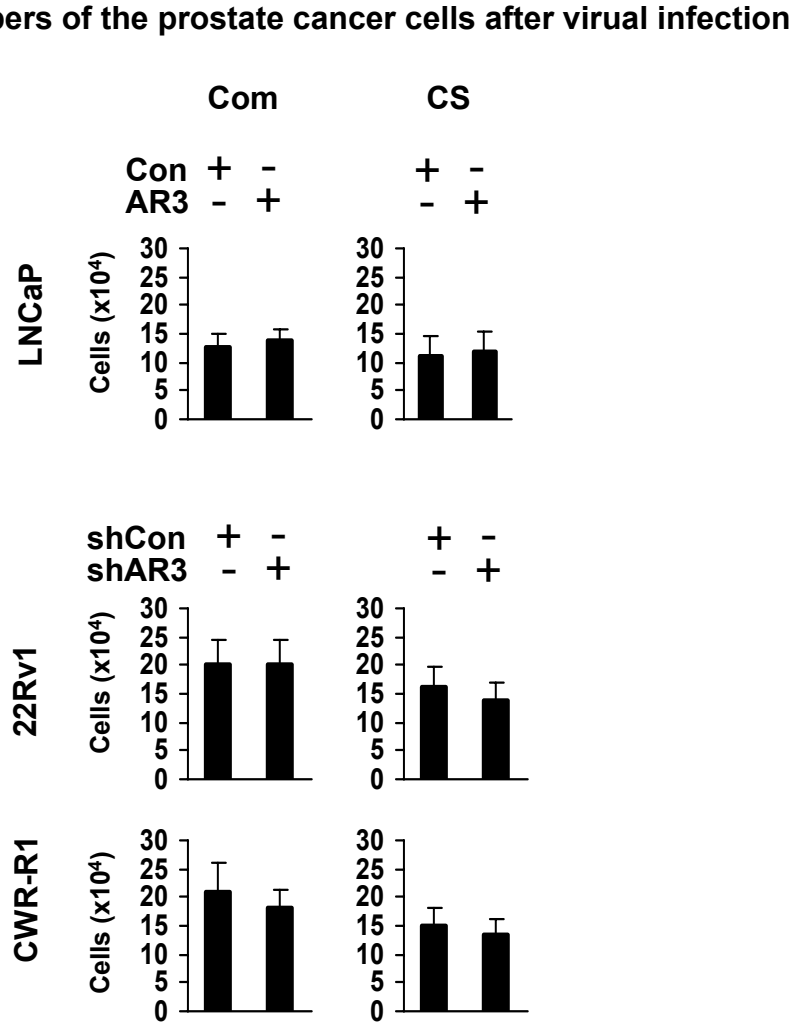
Expression of AR proteins in LNCaP, CWR-R1 and 22Rv1 cells for tumor xenograft experiments before injection. LNCaP cells were infected with *lenti*-virus encoding the AR3 expression vector or the control vector. CWR-R1 and 22Rv1 cells were infected with *lenti*-virus encoding specific shRNA against AR3 (shAR3) or scrambled control shRNA (shCon). At 48 hr postinfection, right before injection of the cells into the castrated mice, the cells were lysed and the cell lysates were blotted with anti-AR and anti-Tubulin antibodies, respectively.

B



Effect of AR3 on PSA expression in LNCaP tumor xenografts. The Western blots of anti-PSA, anti-AR3 and anti-AR of the LNCaP xenograft tumor lysates derived from the castrated SCID mice as described in **Fig.5 B**.

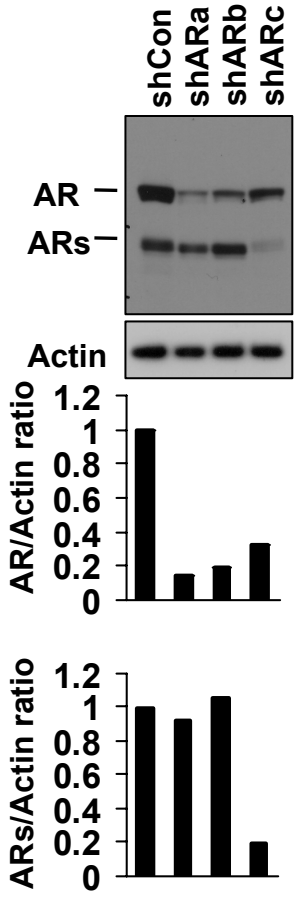
Supplementary Figure 19



In the androgen-depleted (CS) or the regular complete (Com) medium, the LNCaP cells, CWR-R1 and 22Rv1 cells were infected with lentivirus encoding AR3 expression construct (AR3) and the control vector, or AR3 shRNA-1 (shAR3) and control shRNA (shCon) as indicated. At 48 hr after infection, the numbers of the cells were quantified using a hemocytometer and plotted on the bar graph.

Supplementary Figure 20

Quantification of the western-blots in Fig.1 B.



Selective knockdown of AR long/short forms by AR shRNAs. CWR-R1 cells were infected with the *lenti*-virus encoding AR shRNAa, AR shRNAb, AR shRNAc and control shRNA (shARa, shARb, shARc and shCon). The cell lysates were subjected to western blot with anti-AR and anti-Actin antibodies as described in **Fig. 1B**. The AR, ARs and Actin bands were quantified by densitometry analysis. The levels of AR and ARs were normalized by calculating the ratios of AR/Actin and ARs/Actin. The changes in fold compared to the control shRNA (shCon) were plotted at the bottom panels.

Supplementary Table 1 Genes commonly regulated by AR3 and AR

Gene symbol	*Biological process*	*Change upon knockdown*
STK32B	cell signaling	**up**
SYT4	vesicular trafficking and exocytosis	
CPA1	proteolysis, zymogen inhibition	
GIPR	secretion of insulin, anabolic response	
GPR101	cell signaling	
MAGEA10	embryonic development, tumor transformation and progression	
OTOR	cartilage development and maintenance	
ONECUT1	transcriptional activator, liver gene transcription	
IFNA10	inflammatory response	
ZNF224	transcriptional regulation	
NBN	cellular response to DNA damage	
ITIH5	hyaluronan metabolic process	
ZNF624	transcriptional regulation	
DYNLT3	motor activity	
B4GALT4	cell metabolism	
IGFBP3	growth factor signaling	
PNPLA4	lipid metabolism	
PTPRK	regulation of processes involving cell contact and adhesion	
ANKRA2	cell endocytosis	
PCDH10	cell adhesion, mesoderm development	
CD19	B cell receptor signaling pathway	
CDH18	cell adhesion	
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ZNF589	DNA binding protein, transcription regulation	**down**
ANGPTL4	inhibiting vascular activity as well as tumor cell motility and invasiveness	
PDXP	coenzyme for biochemical homeostasis	
CSRP1	neuronal development	
PPP5C	RNA biogenesis and/or mitosis	
TRAF4	adapter protein and signal transducer	
NT5DC3	lipid metabolism	
PPFIA3	disassembly of focal adhesions	
SRF	MAPK signaling pathway	
GAS1	tumor suppressor gene	
LDHA	pyruvate metabolism	
CHRNA2	modulation of ion-conducting channels	
TMEM81	transmembrane cell component	
SLC25A37	protein transportation	
UBE2M	protein-ubiquitination pathway	
DHRS2	reactive carbonyls metabolism	
SNRPB	pre-mRNA splicing or in snRNP structure	
TPM1	striated muscle contraction	
SNAP23	transport vesicle docking and fusion	
FKBP5	protein binding, immunoregulation	
PRKAR2A	protein transport	
HNRPA0	pre-mRNA splicing or in snRNP structure	
TMED9	protein transportation and localization	

Supplementary Table 2 Genes preferentially regulated by AR

Gene symbol	*Biological process*	*Change upon knockdown*
PCDH11Y	cell-cell recognition	**up**
HOXA13	transcription factor, involved in embryonic development	
CDKN1A	cell cycle progression	
PCDH9	neuronal connections and signal transduction	
FAS	apoptosis	
MYBL1	proto-oncogene, transcription activator	
TAF9B	DNA binding, transcription regulation	
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AR	transcription factor	**down**
CLU	inhibit apoptosis	
WNK3	serine-threonine protein kinase, cell signaling	
TCF3	transcription factor in cell differentiation	
IL6ST (gp130)	cytokine signal transduction	
TERT	oncogenesis, cellular senescence	
DACH2	transcriptional cofactor	
TMEPAI	Androgen induced gene	
KLK3	serine proteases, biomarker for prostate cancer	
KLK15	serine proteases, cancer biomarker	
CLDN4	component of cell tight junctions	
CDC2	cell cycle regulation	
WNT3	embryogenesis	
WNT10B	Inhibition of adipogenesis	

Supplementary Table 3 Genes preferentially regulated by AR3

Gene symbol	*Biological process*	*Change upon knockdown*
EPHA3	Ephrin receptor, cell signaling	**up**
AF268194 (IRA2)	cell signaling	
Neuralin-1	BMP (bone morphogenetic protein) pathway	
OPTN	TNF-alpha signaling pathway	
RYR2	Cardiac muscle ryanodine receptor	
SCML2	transcription repression	
RFX3	transcription factor	
AI278811 (MYC like)	transcription factor, proto-oncogene	
EFCBP2	EF-hand calcium binding protein 2	
TES	Zinc finger ion binding protein	
SLC40A1	solute carrier, iron-regulated transporter	
SLC7A11	amino acid transport	
RGMB	cell membrane lipid-anchor	
SYTL2	vesicle trafficking	
APOLD1	angiogenesis	
DMD	actin binding, cytoskeletal anchoring	
NTNG1	neurite outgrowth	
GLUD1	nitrogen metabolism	
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AKT1	Proto-oncogene, Serine/threonine-kinase	**down**
AKT1S1	Akt-mTOR pathway	
WNK1	Serine/threonine-kinase, signal transduction	
MAP4K4	MAP Kinase Pathways	
ADCY6	signal transduction	
PICK1	organize subcellular localization of membrane proteins	
HDAC3	chromatin modification, epigenetic	
SELENBP1	Golgi protein transport	
EVI5	regulator of cell cycle progression	
ELK1	Ets family transcriptional factor, proto-oncogene	
SLC2A4RG	transcription factor	
ARFGAP1	membrane trafficking and vesicle transport	
PRG2	immune response	
PLEKHA3	lipid binding	
DNM2	receptor mediated endocytosis	
MDM4	Proto-oncogene, p53 binding protein	
RAP1GAP	GTPase activity, cell signaling	
CD2BP2	snRNP assembly	
FXR2	RNA binding	
SCUBE1	adhesive molecule	
TPM1	striated muscle contraction	
AP3M1	vesicles trafficking	
KDELR2	endoplasmic reticulum protein trafficking	
RAB6IP1	Rab6-mediated GTPase signaling	
TSR2	signal transduction	
GYS1	glycogen synthase 1, glucose metabolism	
LIMD1	tumor-suppressor gene	
ARNTL2	circadian rhythms	
EMD	nuclear envelope assembling	
METTL7A	methyltransferase, chromatin modification	
HOXB7	transcription factor, inhibition of differentiation	
MDFI	transcription repressor, inhibition of differentiation	
TAF9B	DNA binding, transcription regulation	