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Supplemental Information

Single-Cell Analysis Identifies LY6D as a Marker Linking Castration-Resistant Prostate Luminal Cells to Prostate Progenitors and Cancer

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Figure S1
Related to Figure 1

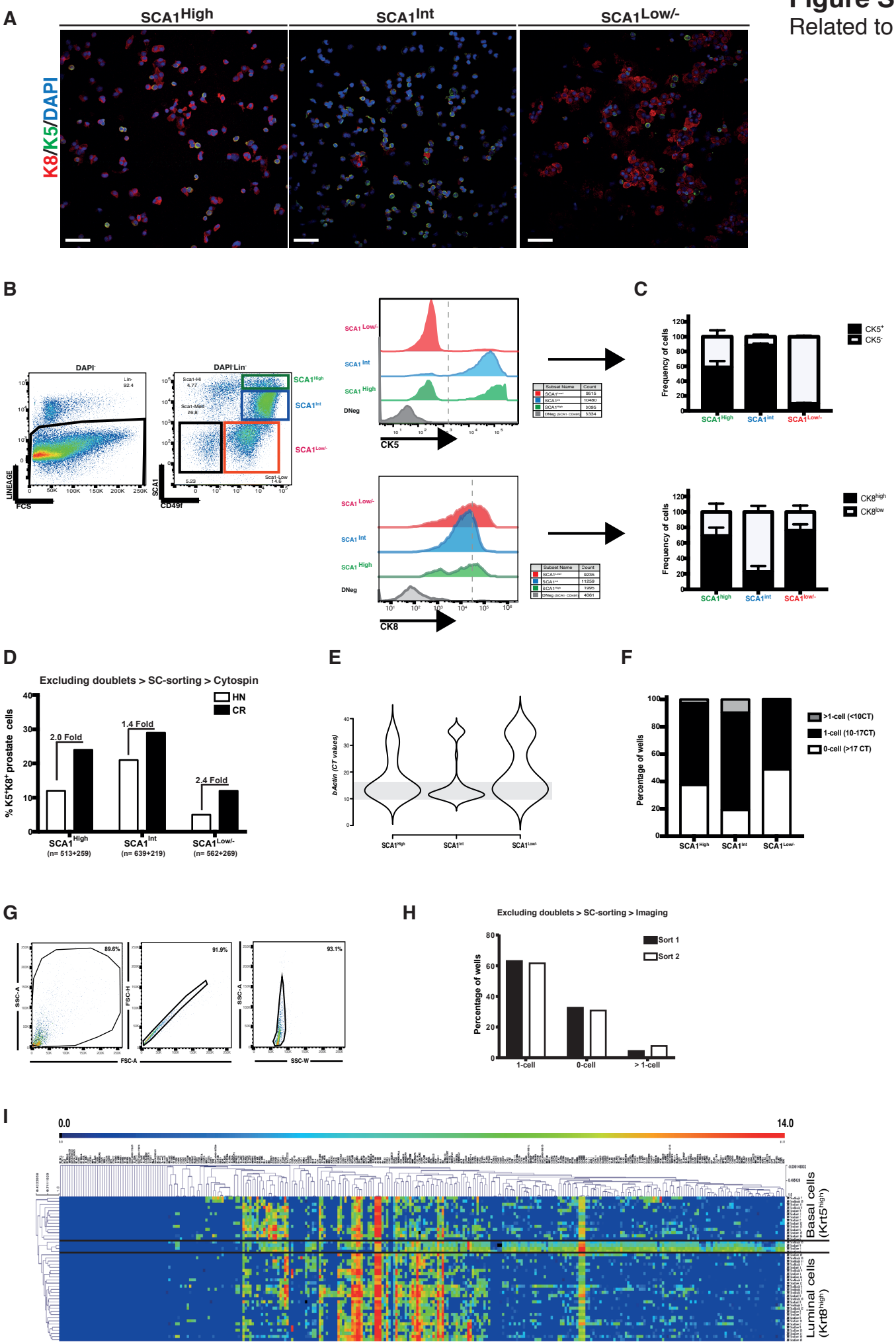


Figure S1. Single cell expression analysis of prostate cells from hormone-naïve (HN) mice (Related to Figure 1).

(A) K5 and K8 co-IF staining of a representative HN prostate sample based on three FACS-sorted populations ($SCA1^{high}$, $SCA1^{int}$, $SCA1^{low/-}$). Nuclei counterstained with DAPI. Scale bars represent 50µm.

(B) FACS analysis of Lin^- cells from microdissected prostates (from HN mice) confirmed K5 and K8 expression patterns in basal-enriched ($SCA1^{int}$), luminal-enriched $SCA1^{high}$ and $SCA1^{low/-}$ cells.

(C) Quantification of keratin 5 (K5) and keratin 8 (K8) marker expression profiles by flow cytometry staining of the FACS-sorted subpopulations shown in **B.** N=3 for each sorted subpopulation. Error bars represent Mean ± S.E.M.

(D) Quantification of $K5^+K8^+$ double-positive cells by IF staining based on three FACS-sorted subpopulations in HN and CR mice. The number of cells counted in each group is based on n=5 mice for the HN group and n=3 mice for the CR group.

(E) Violin plot showing *ß-actin* gene expression (*Actb*) in each sorted single cells based on three FACS-sorted subpopulations ($SCA1^{high}$, $SCA1^{int}$, $SCA1^{low/-}$).

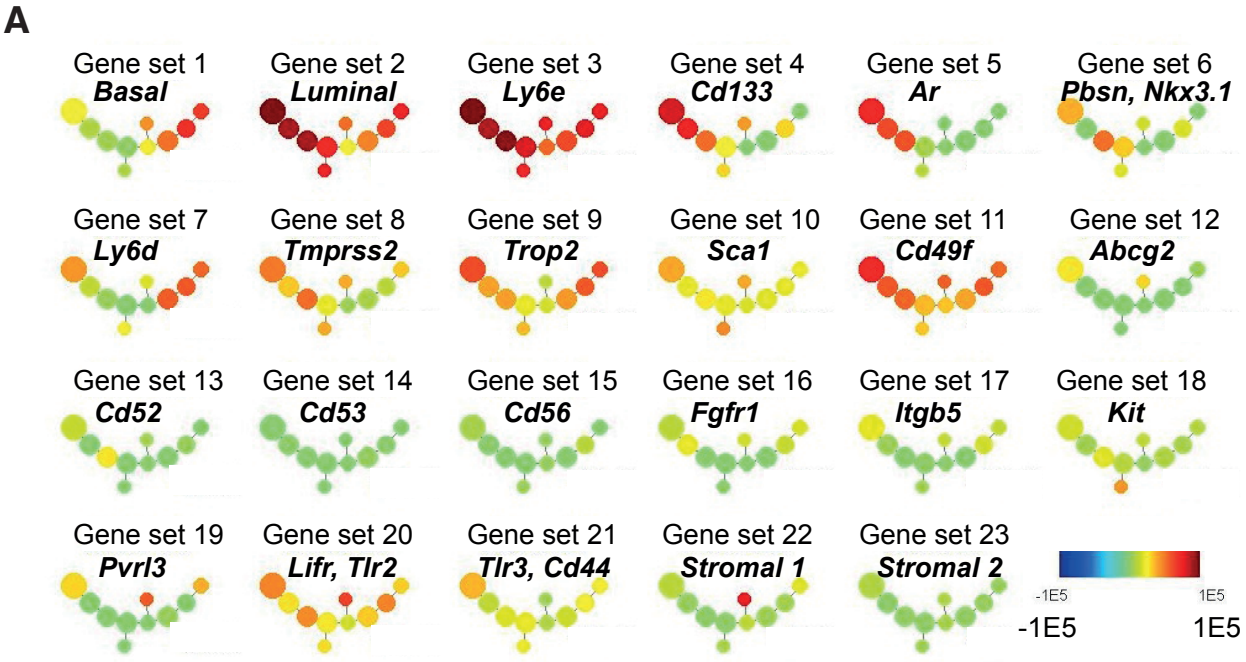
(F) Quantification of *β-actin* CT values. Single cells (1-cell) showed CT values within 10-17 cycles, clumps (>1-cell) CT values >17 cycles, and degraded or no cells (0-cell) CT values <10 cycles.

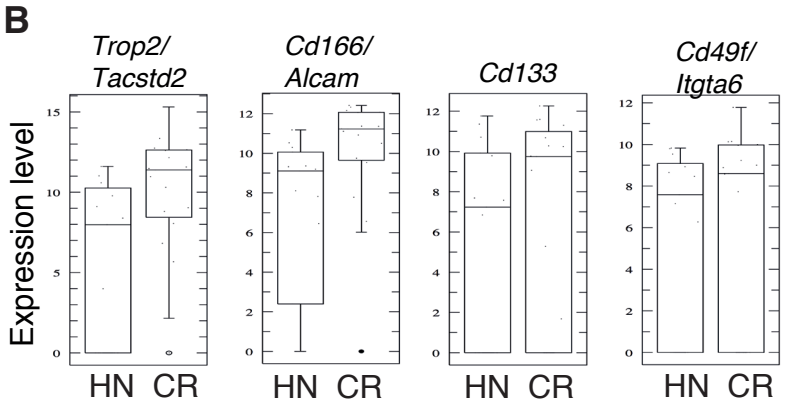
(G) FACS gating strategy used to exclude doublets.

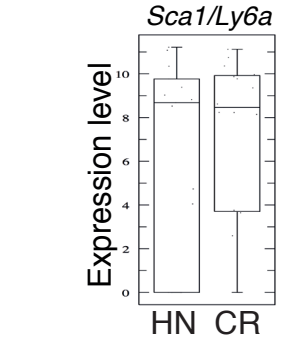
(H) Quantification of cells per well after single-cell sorting onto 96-well plates. The cell number per sorted-well was analyzed from two independent sortings.

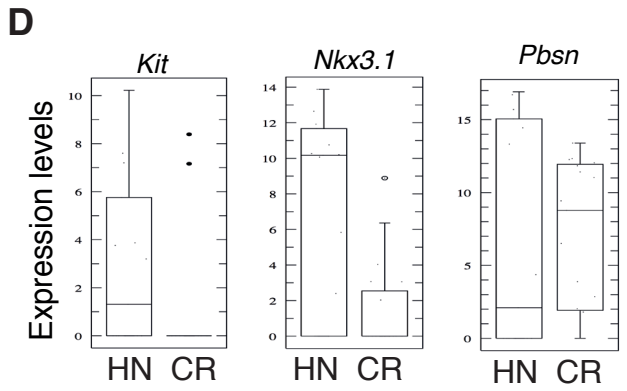
(I) Hierarchical clustering of single prostate cells from HN mice separated as $Krt5^{high}$ basal cells and $Krt8^{high}$ luminal cells. Cell samples in the middle appeared to be outliers as they exhibited abnormal expression levels of housekeeping genes (*Actb*, *Gapdh*) and intermediate expression levels of many other genes.

Figure S2
Related to Figure 2









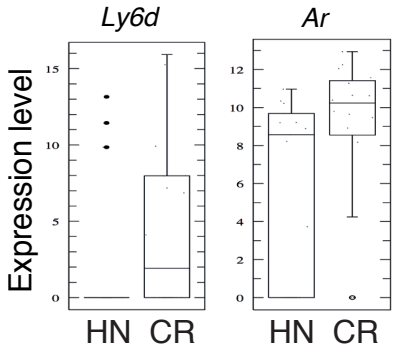


Figure S2. Mapping lineage relation of CR prostate luminal and basal cells to their counterparts from HN mice (Related to Figure 2).

(**A**) Heatmaps showing average expression levels of each gene set in each cell cluster. Color scale is indicated. For each gene set, representative gene(s) or gene type are indicated.

(**B**) Average expression levels of stem/progenitor cell markers *Trop2/Tacstd2, Cd166/Alcam, Cd133/Prom1 and Cd49f/Itgta6* in all single luminal cells (from either HN or CR mice) analyzed in a pooled fashion.

(**C**) Average expression levels of *Sca1/Ly6a* in all single luminal cells (from either HN or CR mice) analyzed in a pooled fashion.

(**D**) Average expression levels of *Kit*, *Nkx3.1, Pbsn* in all single luminal cells (from either HN or CR mice) analyzed in a pooled fashion.

(**E**) Average expression levels of *Ly6d* and *Ar* in all single luminal cells (sorted from the $SCA1^{low/-}$ gate from either HN or CR mice) analyzed in a pooled fashion.

Figure S3

Related to Figures 3 and 4

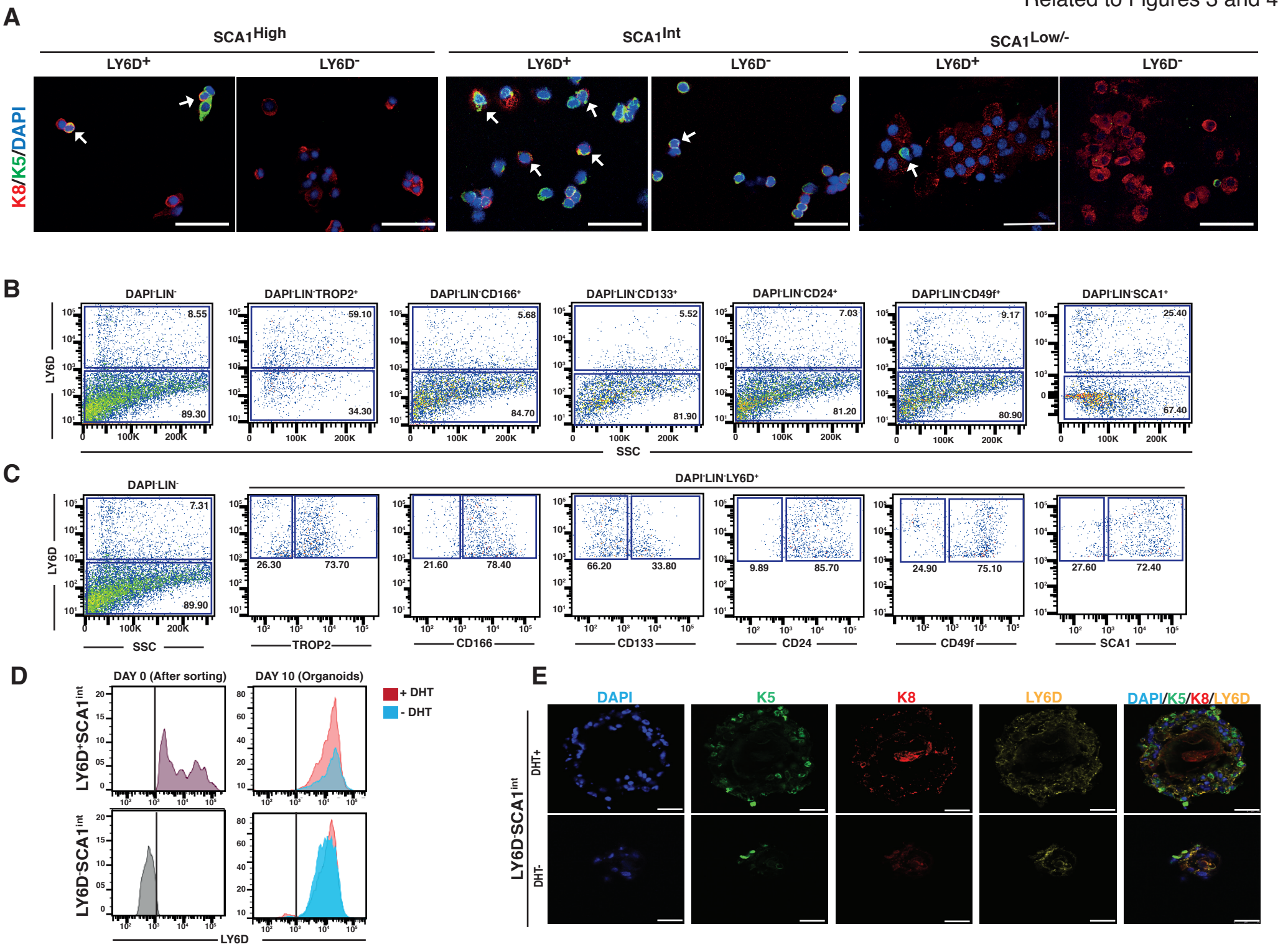


Figure S3. LY6D marks subsets of prostate basal and luminal cells (Related to Figures 3 and 4).

(A) K5 and K8 co-IF staining of a representative HN prostate sample based on $LY6D^{+}$ and $LY6D^{-}$ FACS-sorted subpopulations ($LY6D^{+ \text{ or } -}SCA1^{high}$, $LY6D^{+ \text{ or } -}SCA1^{int}$, $LY6D^{+ \text{ or } -}SCA1^{low/-}$). Arrows indicate $K5^{+}K8^{+}$ double-positive cells. Nuclei counterstained with DAPI. Scale bars represent 50µm.

(B) FACS analysis of prostate cells from microdissected HN mouse prostates showing distribution of $LY6D^{+}$ and $LY6D^{-}$ cells after gating for either Lin^{-} population or $Lin^{-}marker^{+}$ subpopulations (marker: known prostate stem/progenitor markers).

(C) FACS analysis of $Lin^{-}LY6D^{+}$ cells from microdissected HN prostates for their expression of select known prostate stem/progenitor markers.

(D) FACS analysis of LY6D expression in prostate cells sorted from $LY6D^{+}SCA1^{Int}$ (magenta) and $LY6D^{-}SCA1^{Int}$ (grey) gates at day 0 (after FACS-sorting, left), and in cells from organoids (10 days in culture, right) from the indicated subpopulations. Organoids were grown in the presence (Red) or absence (Blue) of 1nM DHT.

(E) Representative co-IF staining results showing LY6D, K8 and K5 staining patterns in organoids from the indicated subpopulation sorted from HN mice. Organoids were initially cultured in the absence of DHT, then stimulated for 7 days with 100nM DHT. Nuclei counterstained with DAPI. Scale bars=25µm.

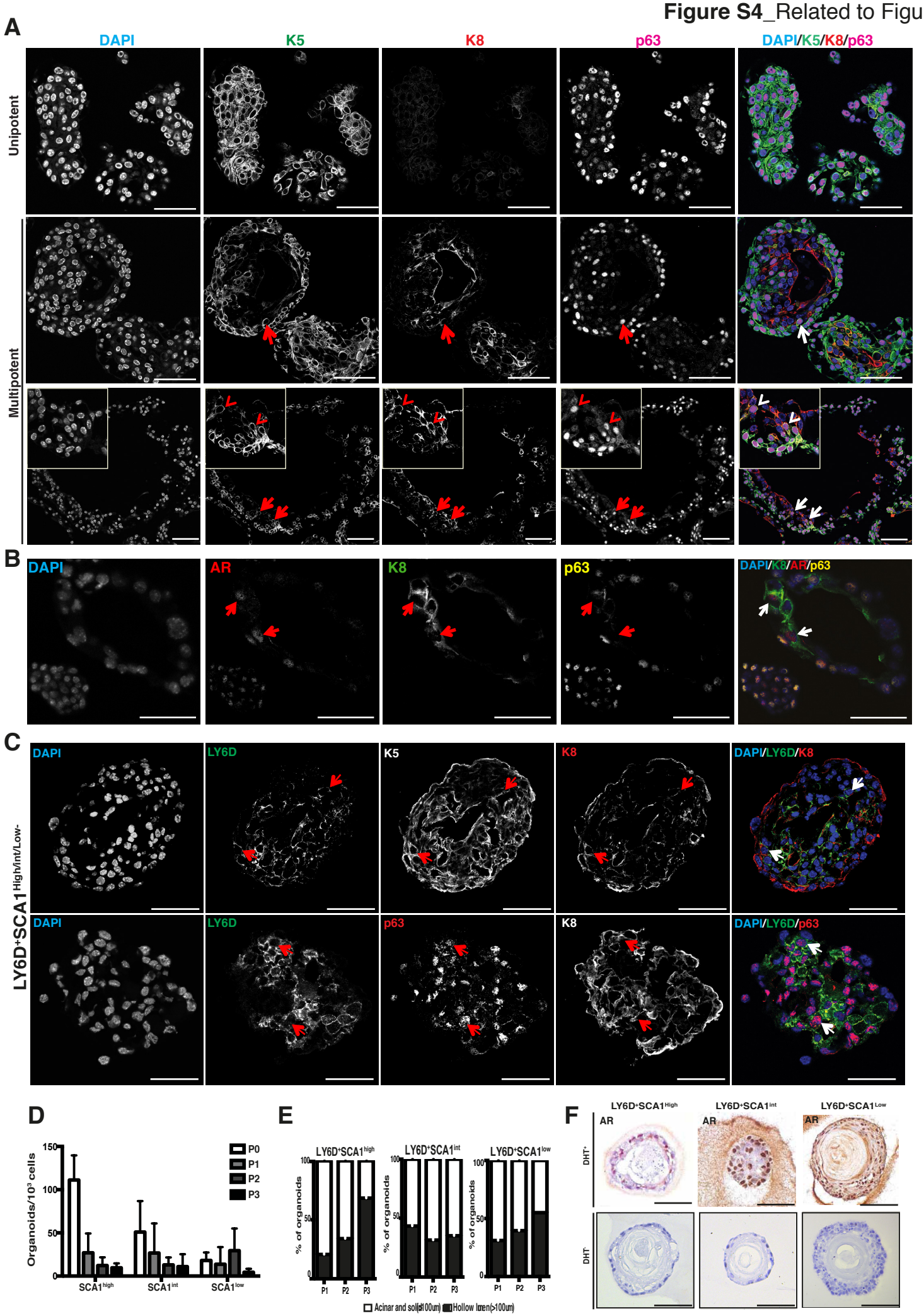

A
DAPI
K5
K8
p63
DAPI/K5/K8/p63
Unipotent
Multipotent
B
DAPI
AR
K8
p63
DAPI/K8/AR/p63
C
LY6D+SCA1High/Int/Low-
DAPI
LY6D
K5
K8
DAPI/LY6D/K8
DAPI
LY6D
p63
K8
DAPI/LY6D/p63
D
Organoids/10³ cells
150
100
50
0
P0
P1
P2
P3
SCA1high
SCA1int
SCA1low
E
LY6D+SCA1high
LY6D+SCA1int
LY6D+SCA1low
% of organoids
100
50
0
P1
P2
P3
Acinar and solid(<100um)
Hollow lumen(>100um)
F
LY6D+SCA1High
LY6D+SCA1Int
LY6D+SCA1Low
AR
DHT+
DHT-

Figure S4. $LY6D^+$ prostate cells are enriched for organoid-forming potential (Related to Figure 4).

(A) Representative co-IF staining results showing K8, K5 and p63 staining patterns in organoids from the $LY6D^+$ $SCA1^{High}$, $SCA1^{Int}$ or $SCA1^{Low/-}$ subpopulation sorted from HN mice. Nuclei counterstained with DAPI. Arrows and arrowheads in insets indicate $K5^+K8^+p63^+$ triple-positive cells. Scale bars=50μm.

(B) Representative co-IF staining results showing AR, K8, and p63 staining patterns in organoids from the $LY6D^+$ $SCA1^{High}$, $SCA1^{Int}$ or $SCA1^{Low/-}$ subpopulation sorted from HN mice. Nuclei counterstained with DAPI. Arrows indicate AR^+K8^+ double-positive cells. Scale bars=50μm.

(C) Representative co-IF staining results showing LY6D, K8, p63 and K5 staining patterns in organoids derived from FACS-sorted $LY6D^+$ (Lin^-SCA1^{high}, Lin^-SCA1^{int} and $Lin^-SCA1^{low/-}$) prostate cells from HN mice. Nuclei counterstained with DAPI. Arrows indicate $LY6D^+K5^+K8^+$ and $LY6D^+K8^+p63^+$ triple-positive cells. Scale bars=25μm.

(D) Serial passage (P) experiments showing organoids formed per 1,000 plated cells during serial passages; cells were originally sorted from the $Lin^-SCA1^{high}LY6D^+$, $Lin^-SCA1^{int}LY6D^{+\text{ or }-}$ and $Lin^-SCA1^{low/-}LY6D^{+\text{ or }-}$ prostate cell subsets from HN mice, respectively.

(E) Percentages of acinar, solid or translucent (hollow lumen) organoids formed at different passages of the organoid-forming experiments shown in (**A**). Note prostate cells sorted from the $LY6D^+SCA1^{High}$ gate formed increasing numbers of luminal type (hollow lumen) organoids during serial replating.

(F) Representative AR staining results in organoids from the $LY6D^+$ $SCA1^{High}$, $SCA1^{Int}$ or $SCA1^{Low/-}$ subpopulation sorted from HN mice in the presence (+) or absence (-) of dihidrotestosterone (DHT). Scale bars=20μm.

Figure S5
Related to Figure 5

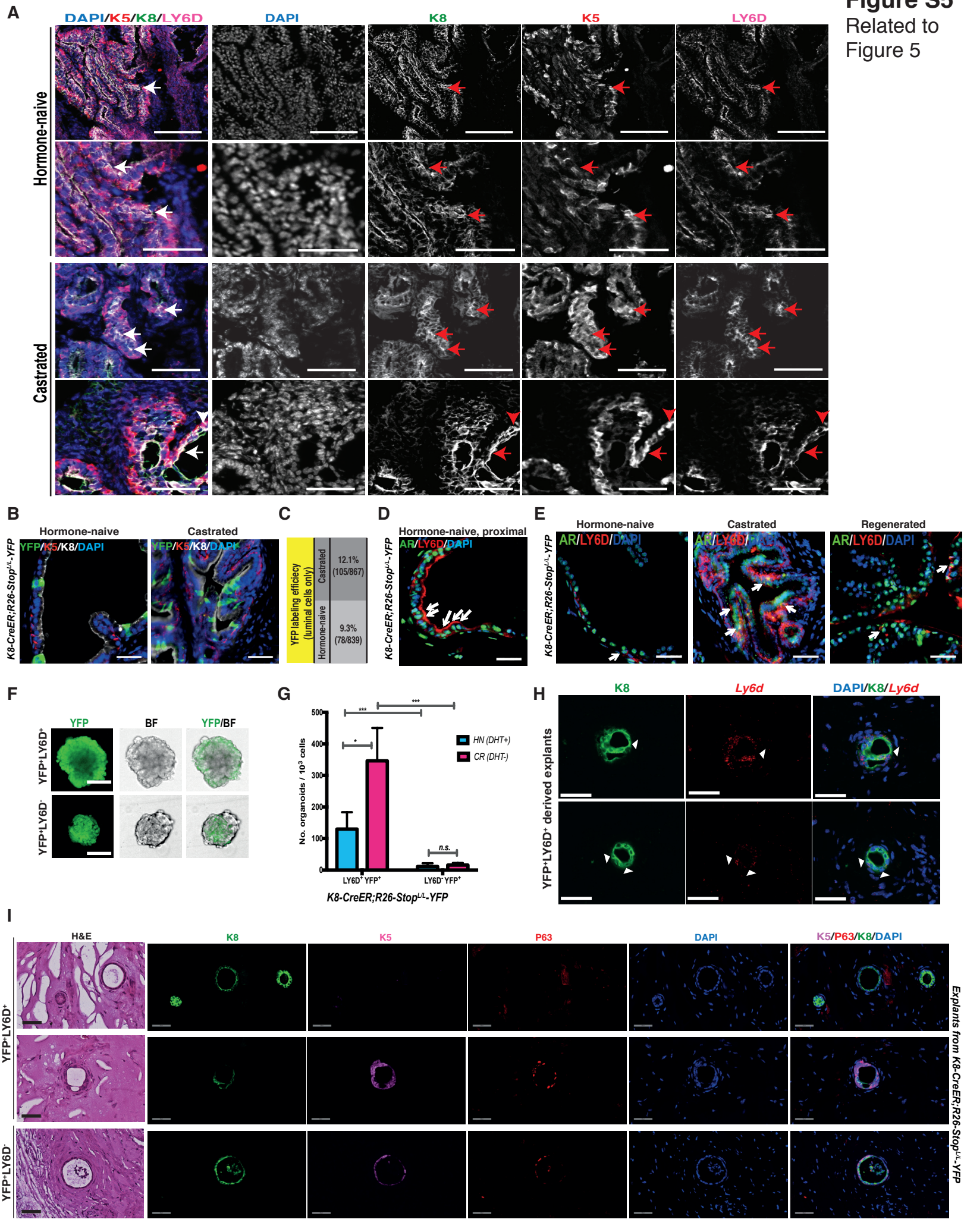


Figure S5. LY6D is a novel marker of CR prostate cells in the luminal lineage (Related to Figure 5).

(A) Representative co-IF staining results showing LY6D, K8 and K5 staining patterns in hormone-naïve (HN, ~4-week of age) and castrated (adult) wild-type males. Nuclei counterstained with DAPI. Red arrows indicate $LY6D^{+}K8^{+}K5^{+}$ cells. Scale bars: top panels=100µm, lower panels=50µm.

(B) Representative co-IF staining results showing YFP, K8 and K5 staining patterns in hormone-naïve (HN) and castrated prostates from *K8-CreER;R26Y* mice after tamoxifen induction. Nuclei counterstained with DAPI. Scale bars=50µm.

(C) Quantification of Cre-induced recombination efficiency and subsequent YFP expression in HN and castrated mice.

(D) Multi-color IF staining of HN prostate showing abundant AR^{+} $LY6D^{+}$ cells in the proximal prostate (arrows). Scale bars represent 50µm.

(E) Multi-color IF staining of intact prostate, or castrated prostates at either the regressed state (middle) or upon regeneration (right) showing abundant AR^{+} $LY6D^{+}$ cells in the castrated prostate but rare AR^{+} $LY6D^{+}$ cells in the intact and regenerated prostates (arrows). Scale bars represent 50µm.

(F) Representative YFP (left) and phase (middle) images of organoids derived from sorted luminal $YFP^{+}LY6D^{+\text{ or }-}$ luminal cells sorted from tamoxifen-induced *K8-CreER; R26Y* male. BF: bright field. Scale bars= 100µm.

(G) Quantification of organoids formed from FACS-sorted prostate $YFP^{+}LY6D^{+\text{ or }-}$ luminal cells from hormone-naïve (HN) and castrated (CR) *K8-CreER;R26Y* males. *P value*: (*) $p<0.05$, (***) $p<0.001$, (n.s) *non-significant.* Error bars represent Mean ± S.E.M.

(H) *Ly6d* expression analysis by *in situ* hybridization (RNAscope, in red) on explants derived from $YFP^{+}LY6D^{+}$ cells. K8, keratin 8 in green; nuclear staining in DAPI. Scale bars= 50µm.

(I) Representative H&E and IF images of prostatic ductal structures formed by $YFP^{+}LY6D^{+\text{ or }-}$ luminal cells *in vivo.* Ducts derived from $YFP^{+}LY6D^{+}$ luminal cells are either bipotent, containing both basal [$K5^{+}P63^{+}$ (pink, red respectively)] and luminal [$K8^{+}$ (green)] cells, or luminal unipotent ($K8^{+}$ $K5^{-}P63^{-}$). DAPI, nuclear staining. Scale bars= 50µm.

Figure S6
Related to Figure 6

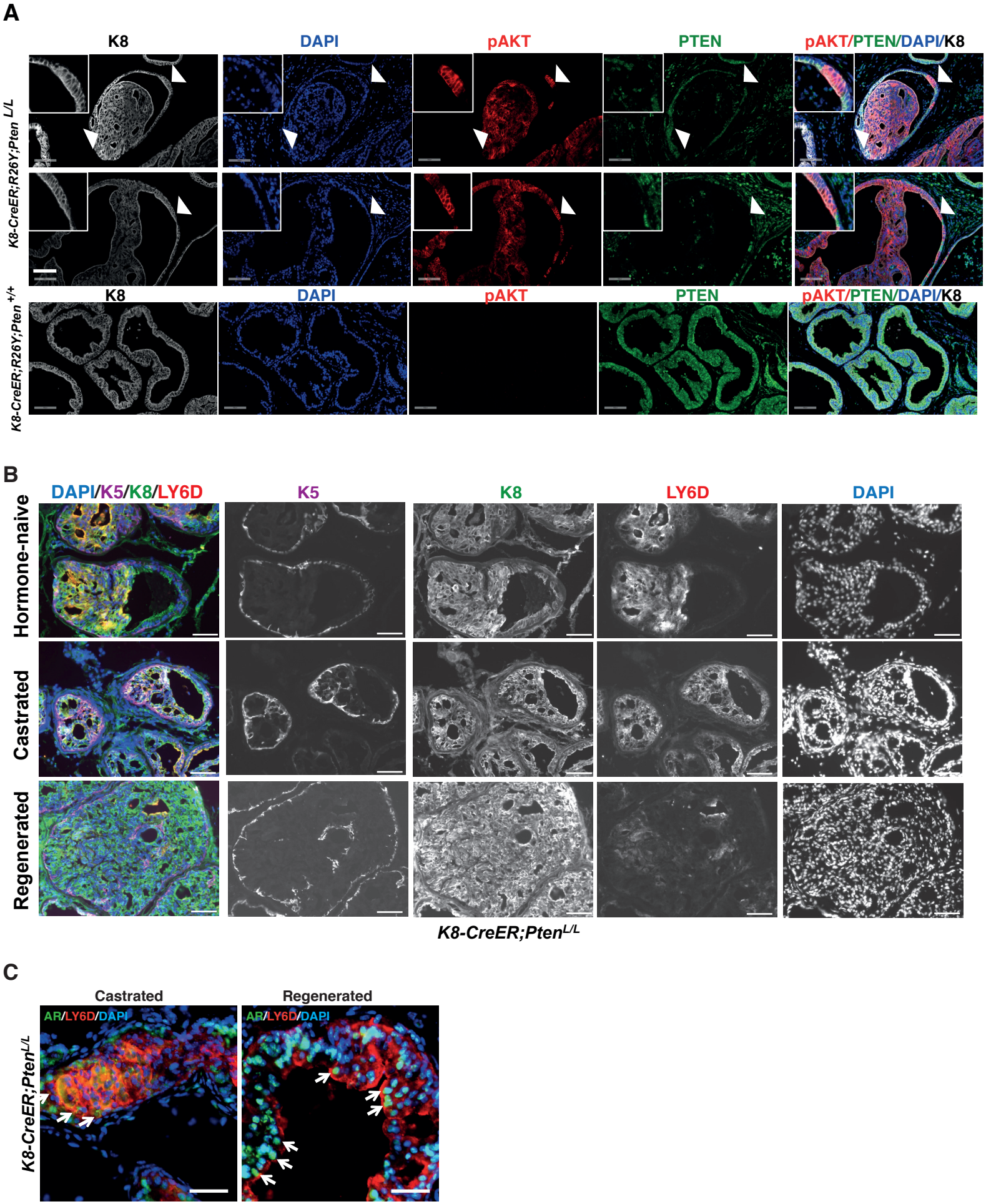


Figure S6. $LY6D^+$ prostate cells are involved in PCa initiation and progression (Related to Figure 6).

(A) IF staining of tamoxifen-induced *K8-CreER;R26Y;Pten*$^{L/L}$ and *K8-CreER;R26Y; Pten*$^{+/+}$ mouse prostate tissue reveals loss of PTEN (green) in the K8-expressing (white) luminal cells. Regions of PTEN loss are indicated by arrowheads. Activation of AKT (red) is observed in the PTEN-negative cells, which is detected by pAKT (Ser473) antibody. DAPI, nuclear staining. Scale bar = 100 μm.

(B) Co-IF staining showing overlaps of $LY6D^+$ cells with $K8^+$ and/or $K8^+K5^+$ cells in prostate sections from either hormone-naïve (HN), or castrated, or regenerated *K8-CreER;Pten*$^{L/L}$ mice. For HN mice, *Pten*-loss was induced at the HN stage; for castrated and regenerated mice, *Pten*-loss was induced at the castrated stage. Scale bars represent 50μm.

(C) Co-IF staining showing abundant AR^+LY6D^+ cells in the prostate from castrated and regenerated *K8-CreER;Pten*$^{L/L}$ male mice (*Pten*-loss induced at the castration stage). The section from the castrated mouse showed largely cytoplasmic staining for AR (left, arrows). The section from the regenerated mouse showed strong nuclear AR staining (right, arrows). Scale bars represent 50μm.

Figure S7
Related to Figure 7

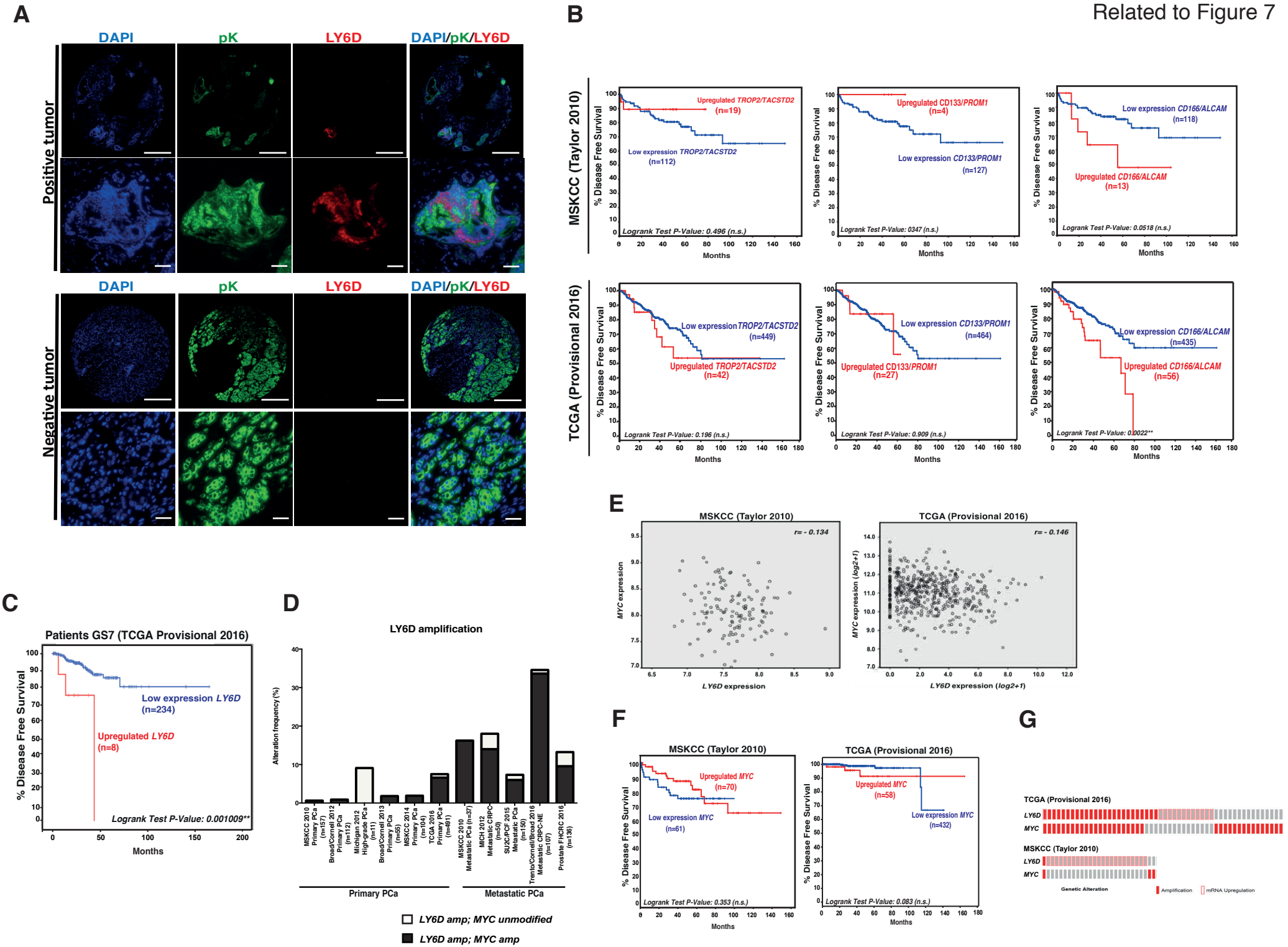


Figure S7. Comparison of LY6D with other markers for correlation with PCa patient outcomes (Related to Figure 7).

(A) Representative IF staining results showing pan-Keratin (pK, epithelial marker) and LY6D staining in human PCa samples. LY6D protein quantification was performed using Definiens software. LY6D positive tumors: cases with detectable $LY6D^+$ prostate cancer cells per core. LY6D negative tumors: cases with no detectable expression of $LY6D^+$ prostate cancer cells. Scale bars=200µm (top panels, 7X), and 20µm (bottom panels, 40X).

(**B**) Kaplan-Meier curve of human primary PCa cases from MSKCC cohort (Taylor et al., 2010) and TCGA cohort (Cancer Genome Atlas Research Network, 2015) analyzing time to biochemical recurrence from diagnosis. Red line depicts patients with upregulated *TROP2/TACSTD2*, *CD133/PROM1* or *CD166/ALCAM* levels [mRNA (z-score>2)], whereas the blue line depicts patients with low expression levels of the indicated stem/progenitor markers.

(C) Kaplan-Meier curve of human primary PCa cases from TCGA cohort (Cancer Genome Atlas Research Network, 2015) analyzing Gleason score 7 patients' disease-free survival. Red line depicts patients with high levels of *LY6D* expression [mRNA (z-score>1.3)], whereas the blue line depicts patients with low levels of *LY6D*.

(D) Frequencies of *LY6D* gene amplification in PCa cohorts from cBioportal, including the Broad/Cornell(Baca et al., 2013; Barbieri et al., 2012), Michigan(Grasso et al., 2012), MSKCC(Hieronymus et al., 2014), TCGA(Cancer Genome Atlas Research Network, 2015), SU2C/PCF(Robinson et al., 2015), the Trento/Cornell/Broad CRPC-NE (Beltran et al., 2016) and the Fred Hutchinson CRC (Kumar et al., 2016) cohorts. White depicts *LY6D* gene amplification without MYC amplification. Black depicts *LY6D* and *MYC* amplification.

(E) Correlation between *LY6D* and *MYC* mRNA expressions in primary prostate tumors (MSKCC cohort (Taylor et al., 2010) n=131, TCGA cohort (Cancer Genome Atlas Research Network, 2015) n=498).

(F) Kaplan-Meier curve of human primary PCa cases from the MSKCC (Taylor et al., 2010) and TCGA (Cancer Genome Atlas Research Network, 2015) cohorts analyzing time to biochemical recurrence from diagnosis. Red line depicts patients with upregulated levels of *MYC* [mRNA (z-score>2)], whereas the blue line depicts patients with low levels of *MYC* expression.

(G) Pattern of *LY6D* amplification and overexpression in the MSKCC (Taylor et al., 2010) and TCGA (Cancer Genome Atlas Research Network, 2015) cohorts.