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Supplementary Materials for

Delta-like protein 3 expression and therapeutic targeting in neuroendocrine prostate cancer

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Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/11/484/eaav0891/DC1)

Data file S1 (Microsoft Excel format). Tumor measurements for in vivo studies.

Supplementary Materials

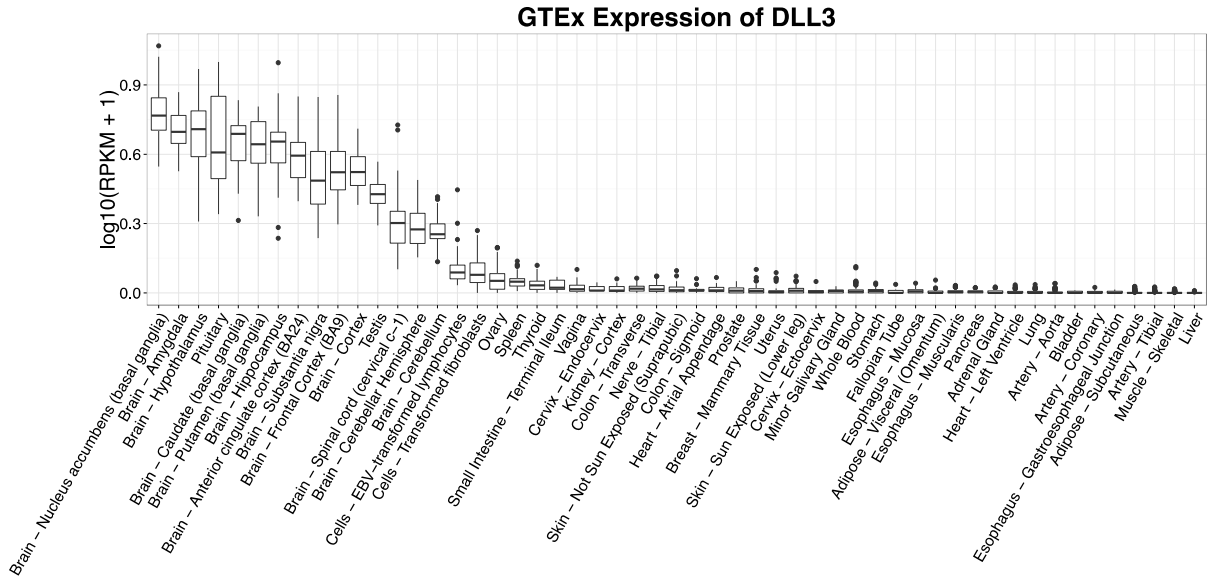


Fig. S1. Genotype-Tissue Expression (GTEx) expression of DLL3 in adult tissues.

Expression of DLL3 in multiple adult tissues evaluated using the GTEx database.

Expression values are presented as $\log_{10}$ (RPKM+1).

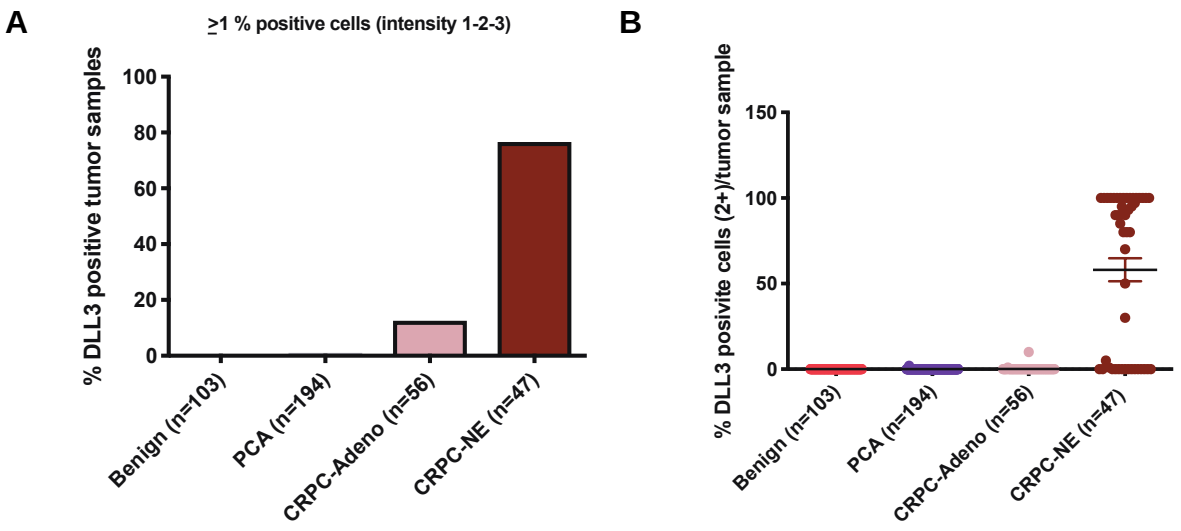


Fig. S2. Evaluation of DLL3-positive cells per tumor. A. Prostate cancer tumors from different categories: Benign (n=103), PCA (n=194), CRPC-Adeno (n=56), and CRPC-NE (n=47) evaluated by IHC as percentage of DLL3 positive tumor samples. **B.** Prostate samples from different categories (benign n=103), PCA (n=194), CRPC-Adeno (n=56), and CRPC-NE (n=47) were evaluated as percentage of DLL3 positive cells with intensity ≥2 per tumor sample. Mean value for CRPC-Adeno was 0.1964% of positive cells per case. Mean value for CRPC-NE was 58.11% of positive cells per case. T-test was performed ($p<0.0001$). The error bars indicate the SEM.

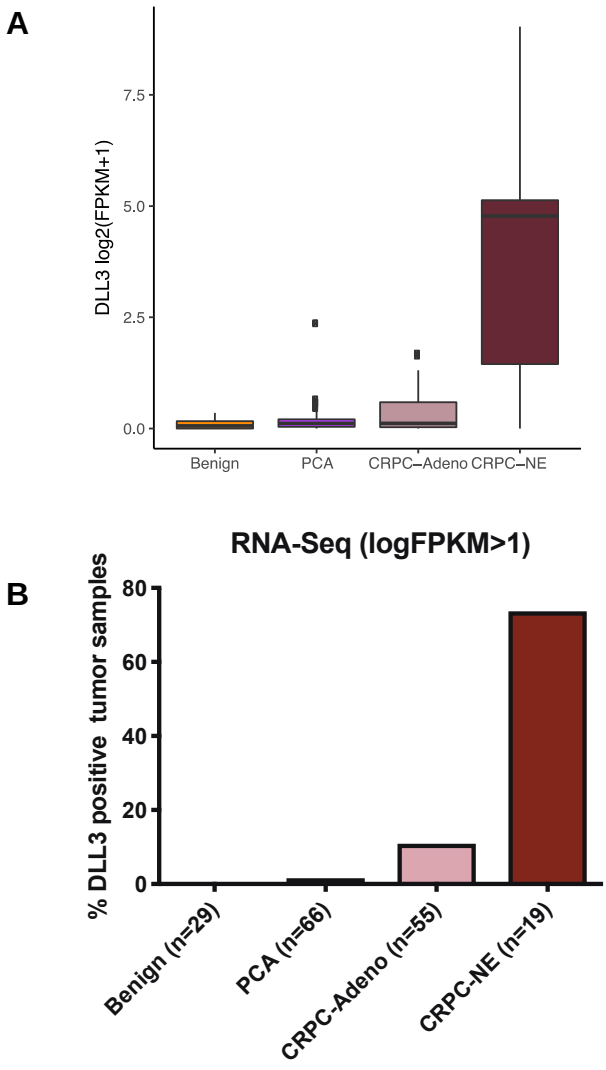


Fig. S3. *DLL3* expression in patient with prostate cancer cohort by RNA-seq. A. *DLL3* expression by RNA-Seq evaluated as $\log_2$ (FPKM+1) across multiple disease stages. Mann-Whitney test was performed: CRPC-NE vs CRPC-Adeno p = 2.488e-05, CRPC-NE vs PCA p = 1.432e-05, CRPC-NE vs benign p = 1.392e-05. **B.** Percentage of *DLL3* positive samples evaluated by RNA-Seq across benign (n=29), PCA (n=66), CRPC-Adeno (n=55), and CRPC-NE (n=19).

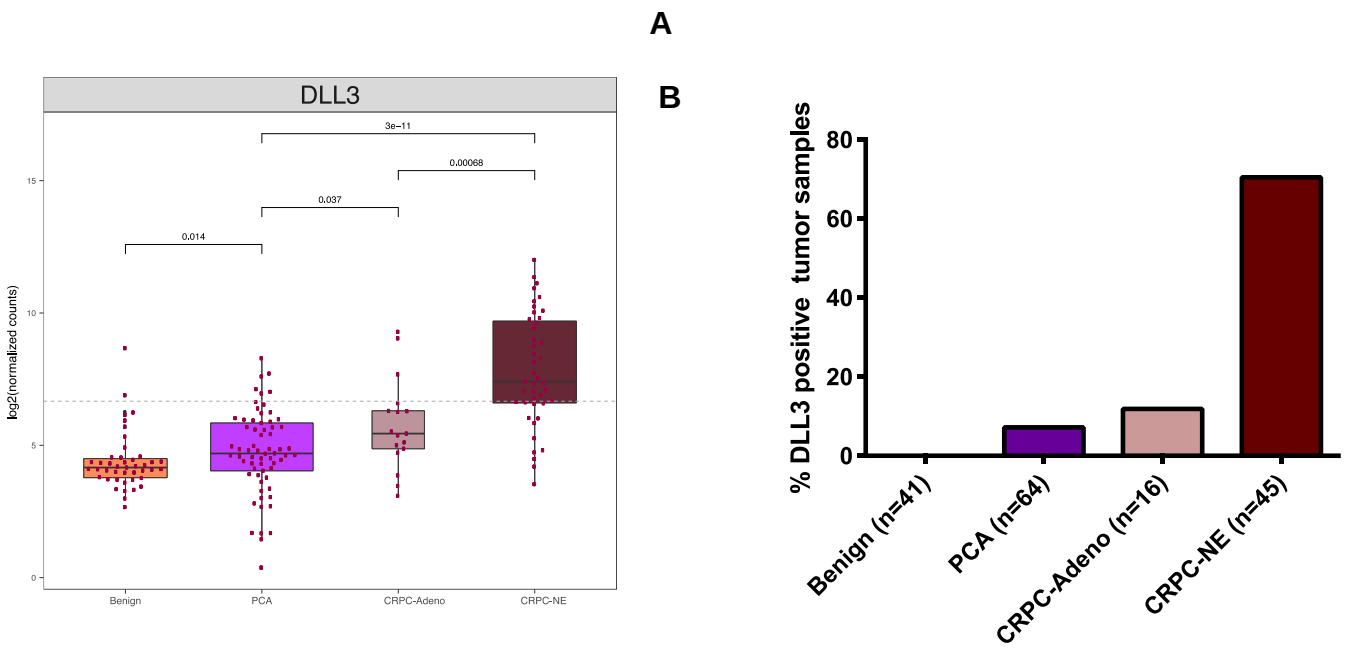


Fig. S4. *DLL3* expression in prostate cancer cohort evaluated by NanoString. A. *DLL3* expression evaluated using a custom-designed nanoString panel. Positive cases are considered with values higher than the mean + IQR of the negative controls with the highest maximum value (cut off= 6.667066). This cut off is represented by the dashed line. Wilcoxon test was performed. **B.** Percentage of *DLL3* positive cases evaluated by nanoString across benign (n=41), PCA (n=64), CRPC-Adeno (n=16), and CRPC-NE (n=45) samples.

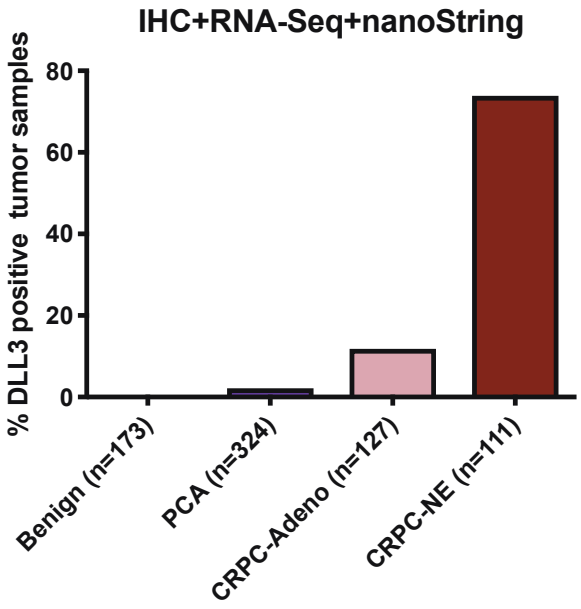


Fig. S5. DLL3-positive tumors evaluated by IHC, RNA-seq, and NanoString.

Percentage of DLL3 positive cases evaluated by RNA-Seq, IHC, and nanoString across benign (n=173), PCA (n=324), CRPC-Adeno (n=127), and CRPC-NE (n=111).

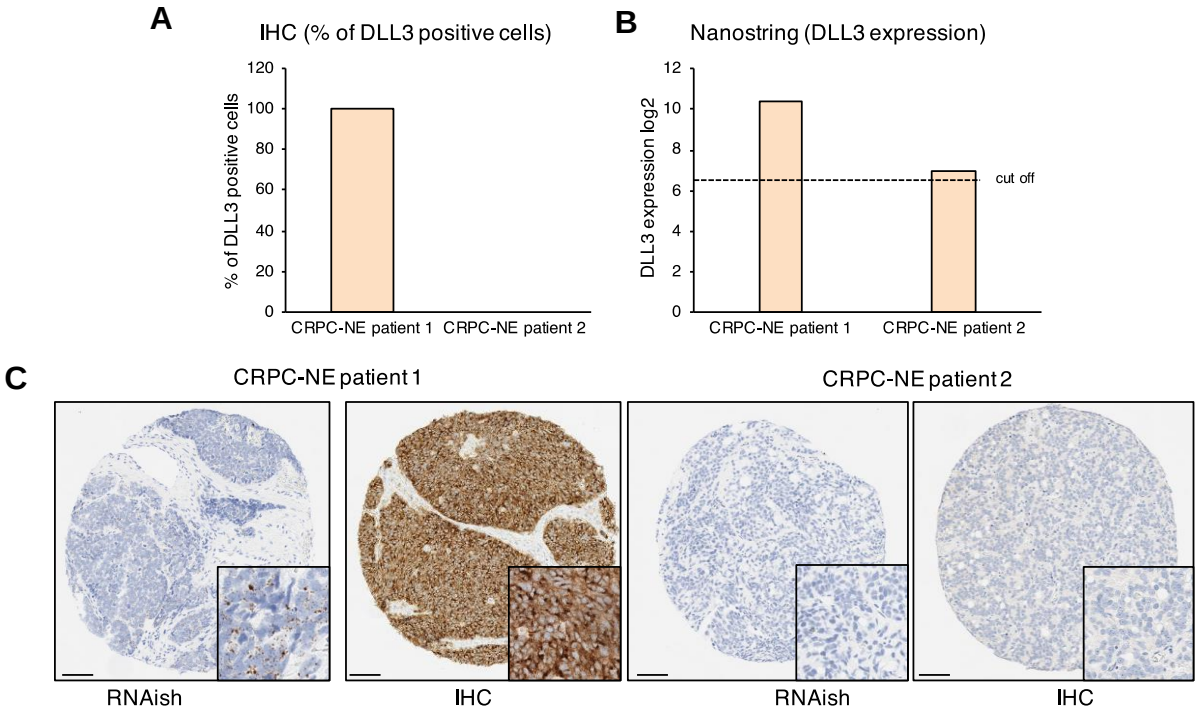


Fig. S6. Representative images of DLL3 expression. A. Percentage of DLL3 positive cells in two representative CRPC-NE patients**. B.** DLL3 expression evaluated as log_2 in two representative CRPC-NE patients. Cut off of DLL3 expression is indicated by a dashed line (cut off $\geq$ the mean + IQR of the negative controls with the highest maximum value) (cut off= 6.667066) **C.** Representative IHC and RNAish expression of DLL3 in CRPC-NE patient 1 (expressing DLL3 by IHC and nanoString) and in CRPC-NE patient 2 (expressing DLL3 by nanoString but not IHC) (scale bar: 200 µm).

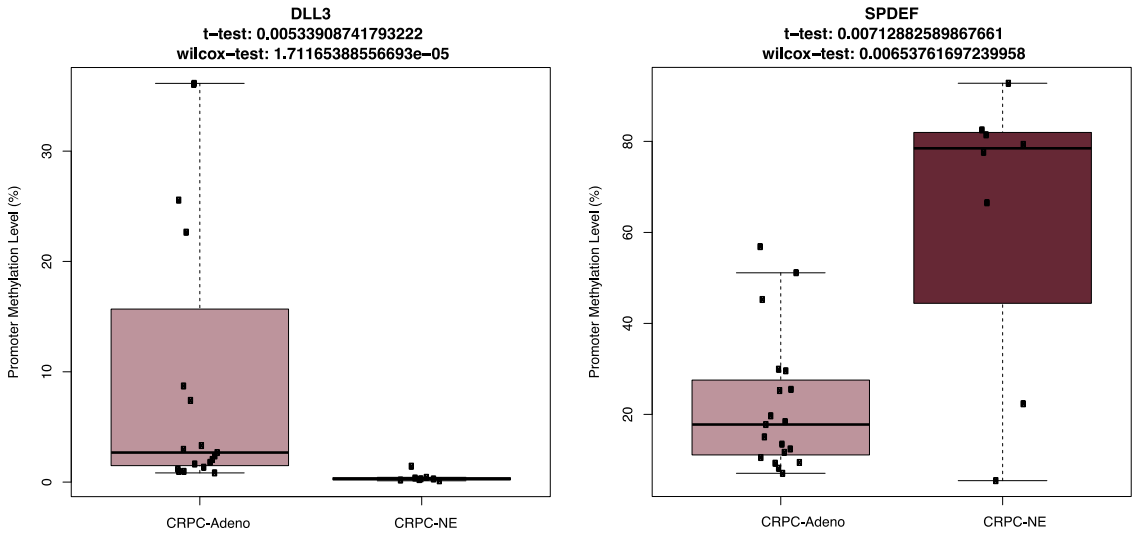


Fig. S7. *DLL3* promoter methylation. Promoter methylation analysis of *DLL3* in CRPC-Adeno and CRPC-NE patients. Wilcoxon test p=1.71e-5 for *DLL3*. *SPDEF* promoter hypermethylation in CRPC-NE was used as control.

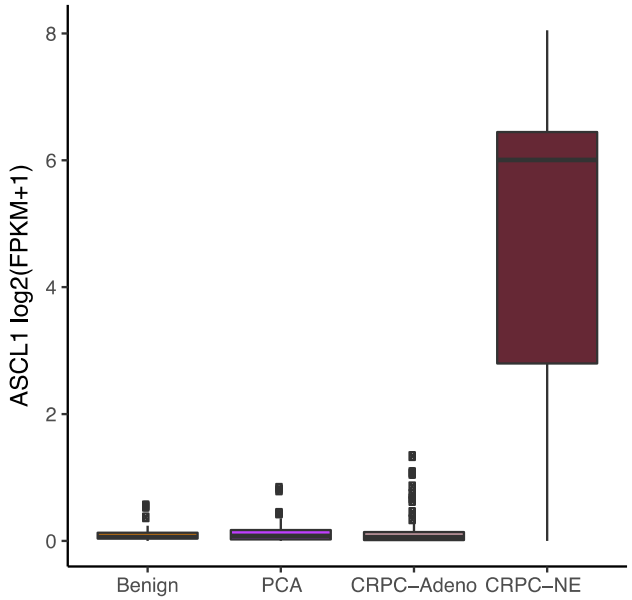


Fig. S8. *ASCL1* expression evaluated by RNA-seq. *ASCL1* expression in prostate cancer patient cohort evaluated as $\log_2$ (FPKM+1) across multiple disease stages. Mann-Whitney test was performed. CRPC-NE vs CRPC-Adeno p = 8.957e-07, CRPC-NE vs PCA p = 1.18e-06, CPRC-NE vs benign p = 8.911e-06.

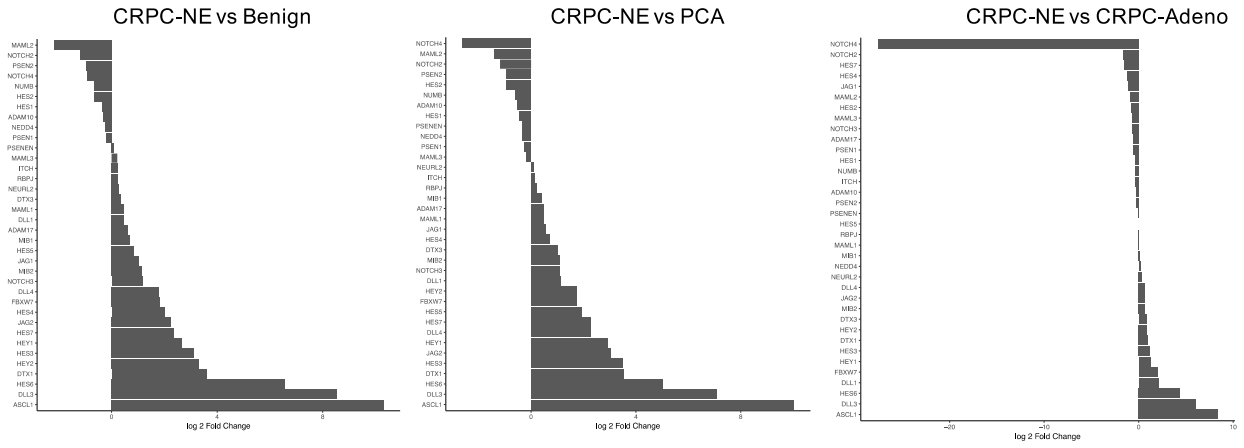


Fig. S9. Differentially expression analysis of Notch signaling genes. Differentially expressed Notch signaling genes for CRPC-NE vs benign, CRPC-NE vs PCA, and CRPC-NE vs CRPC-Adeno evaluated as $\log_2$ fold change. *DLL3* and *ASCL1* were the most differentially expressed Notch signaling genes in CRPC-NE versus benign (p= 6.39E-21 for *DLL3*; p= 4.51E-30 for *ASCL1*), in CRPC-NE versus PCA (p= 3.40E-21 for *DLL3*; p= 2.71E-33 for *ASCL1*), and in CRPC-NE vs CRPC-Adeno (p= 1.49E-11 for DLL3 p= 2.11E-19 for *ASCL1*). We also found *NOTCH2* differentially expressed in CRPC-NE compared to both PCA (p= 0.03185975) and CRPC-Adeno (p= 0.02835248), and *NOTCH4* (p= 3.86E-21) differentially expressed in CRPC-NE vs CRPC-Adeno.

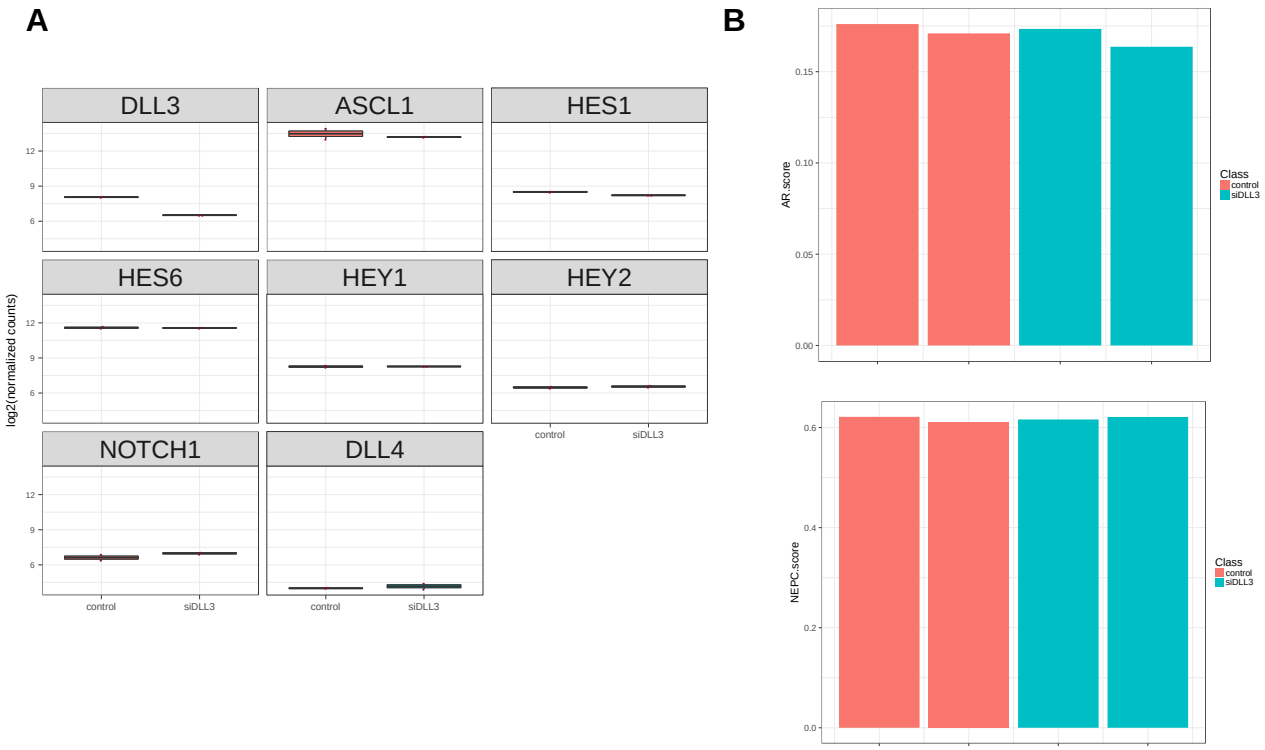


Fig. S10. Silencing *DLL3* in the NCI-H660 cell line. A. Evaluation of mRNA expression of genes involved in Notch pathway using the nanoString platform in siDLL3 (light blue) versus siControl (orange) in NCI-H660 cell line. **B.** NEPC and AR signaling scores as defined in (2) in NCI-H660 transfected with siDLL3 vs siControl.

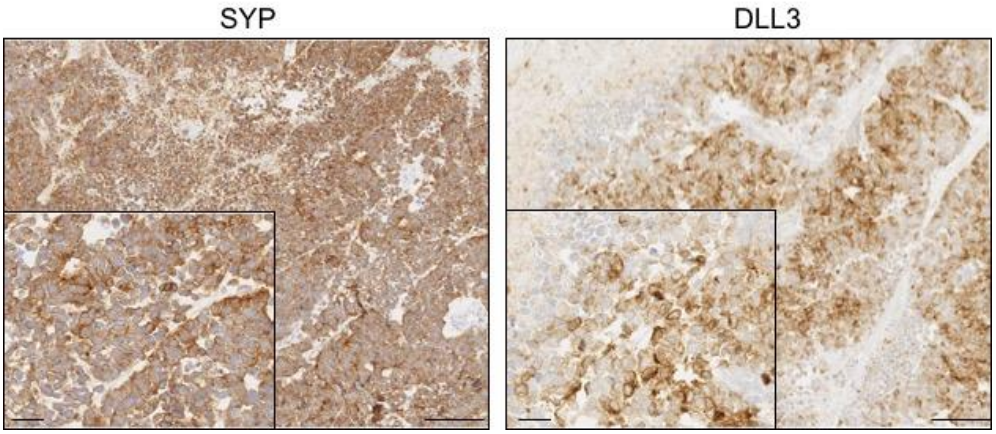


Fig. S11. Correlation between DLL3-positive and DLL3-negative cells in a tumor biopsy. Representative images of synaptophysin (SYP) and DLL3 immunohistochemistry are shown (scale bar: 200 µm, inset: 50 µm). Positive staining for DLL3 is observed in 80% of tumor cells. Positive staining for SYP is observed in 100% of tumor cells.

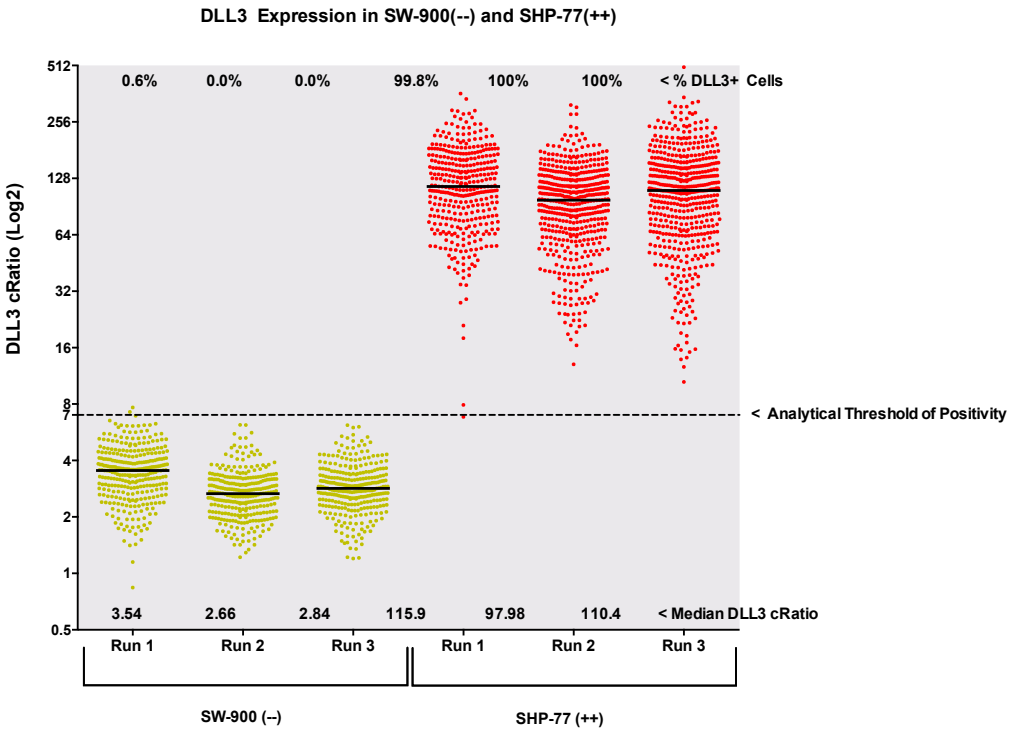


Fig. S12. DLL3 expression cutoff in CTCs. DLL3-negative (in yellow SW-900, squamous cell lung carcinoma) and DLL3 positive cells (in red SHP-77, small cell lung cancer) were used in three independent experiments (Run1 -Run 2-Run 3) to define the cut-off of DLL3 expression in CTCs. DLL3 cRatio (signal-to-noise ratio) is plotted along the y-axis, whereas the 3 experiments with the two cell lines and the means of DLL3 cRatio are indicated on the x-axis. % of DLL3+ cells are indicated on the top of the dot plot for each experiment and for each cell line.

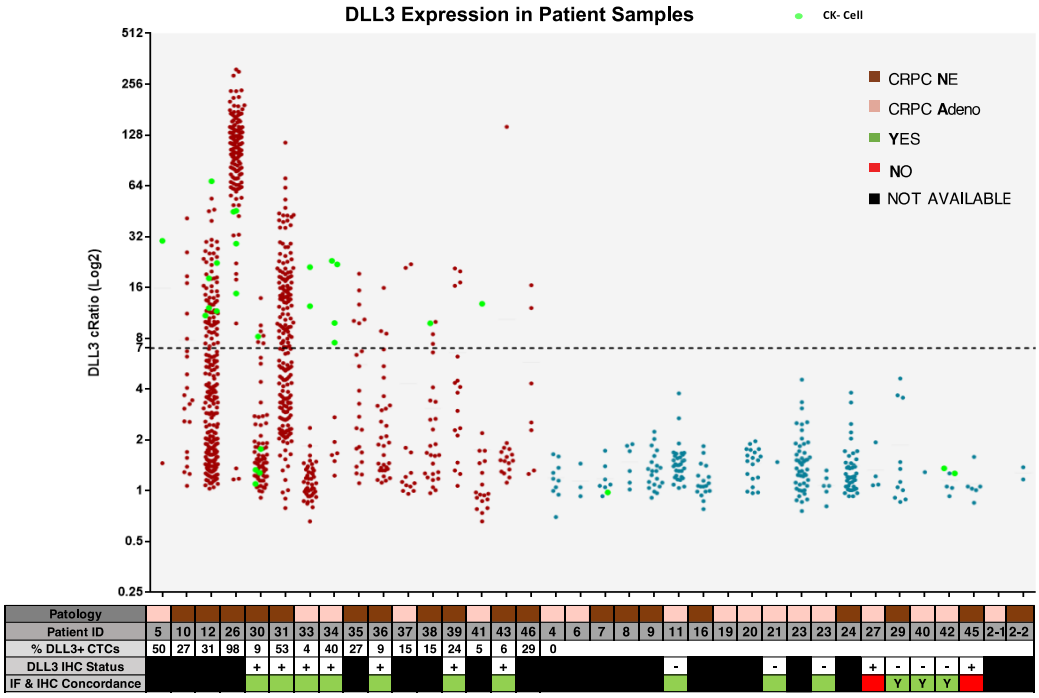


Fig. S13. CK expression in DLL3-positive and DLL3-negative CTCs. Graph showing CK- cells (green) among DLL3- and DLL3+ CTCs of 36 CRPC-Adeno and CRPC-NE patients tested with the DLL3 Epic 4-color immunofluorescence (IF) assay. DLL3 cRatio (signal-to-noise ratio) is plotted along the y-axis, and patient ID is plotted along the x-axis. A table with additional patient information is included (CRPC-NE - brown or CRPC-Adeno - light brown).

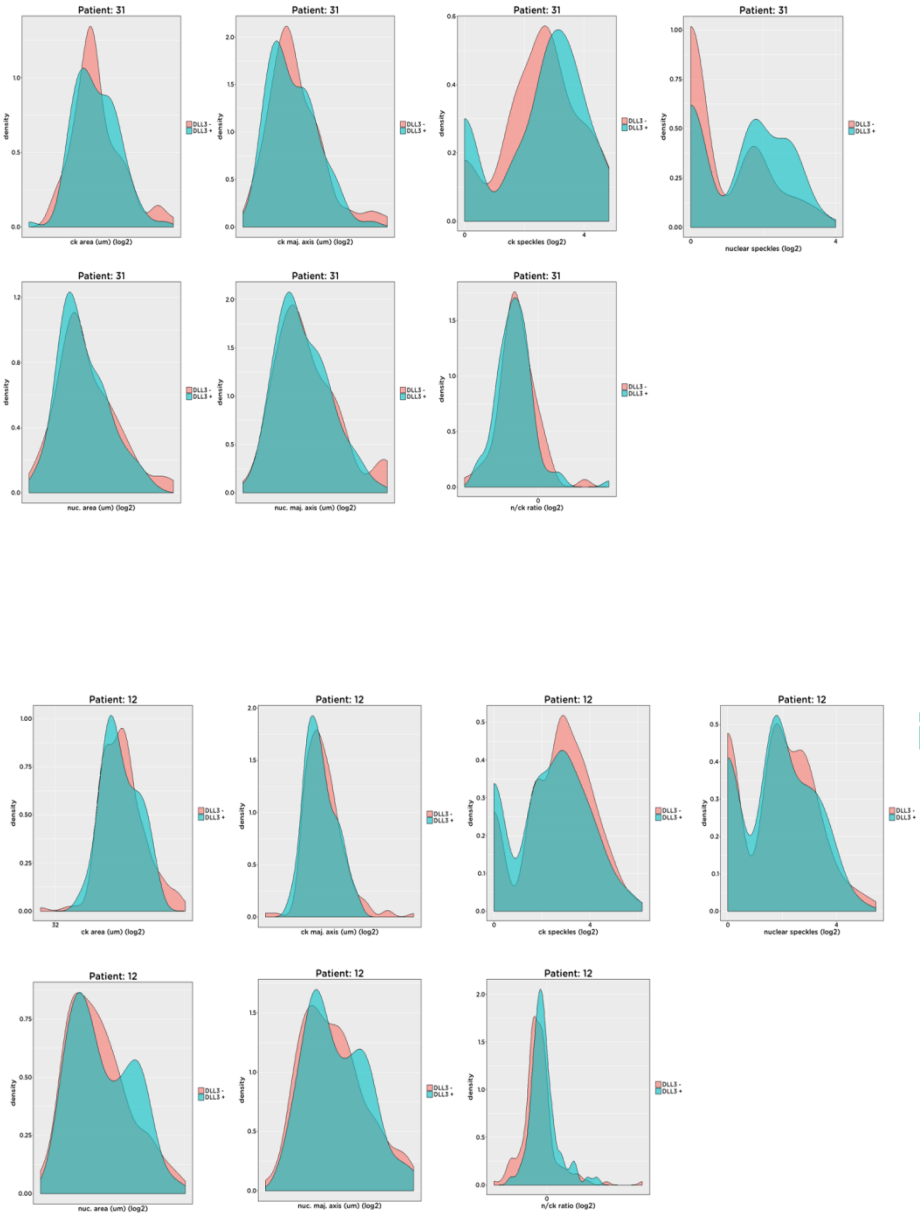
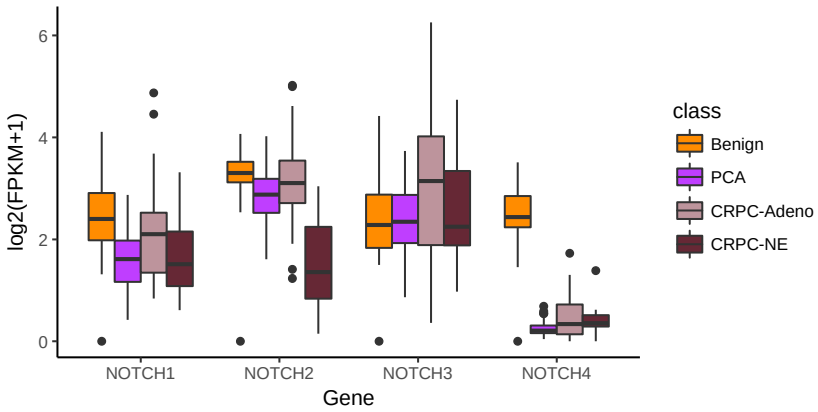


Fig. S14. Correlation between DLL3-positive and DLL3-negative cells in CTCs. Density plots of morphological features in DLL3-positive and DLL3- cells in Patients 31 and 12. The cellular morphological features evaluated in this comparative analysis included: nuclear area, nuclear speckles, nuclear major axis, cytoplasmic area, cytoplasmic speckles, cytoplasmic major axis, and nuclear/cytokeratin ratio (20).

A



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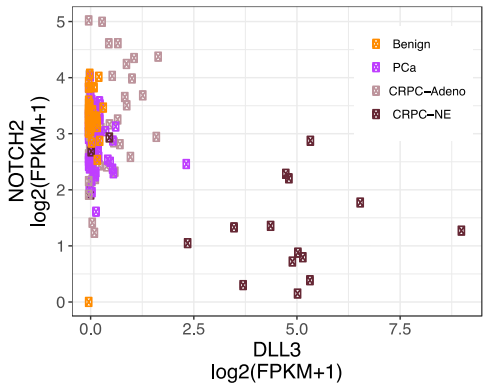

Fig. S15. Expression of Notch receptors across subtypes of prostate cancer and correlation with DLL3 expression. A. Expression of Notch receptors across different subtypes of prostate cancer by RNA-Seq (log2(FPKM+1)). CRPC-NE and CRPC-Adeno were compared using Mann-Whitney U-test (NOTCH1: p-value = 0.04773, NOTCH2: p-value = 2.313e-07, NOTCH3: p-value = 0.2986, NOTCH4: p-value = 0.950).

B. Correlation of *DLL3* and *NOTCH2* expression (log2FPKM+1) in benign, PCA, CRPC-Adeno, and CRPC-NE patients evaluated by RNA-Seq (Spearman correlation with in CRPC-NE samples rho = -0.4263, p = 0.0701; in all samples rho = -0.18414, p = 0.0165).

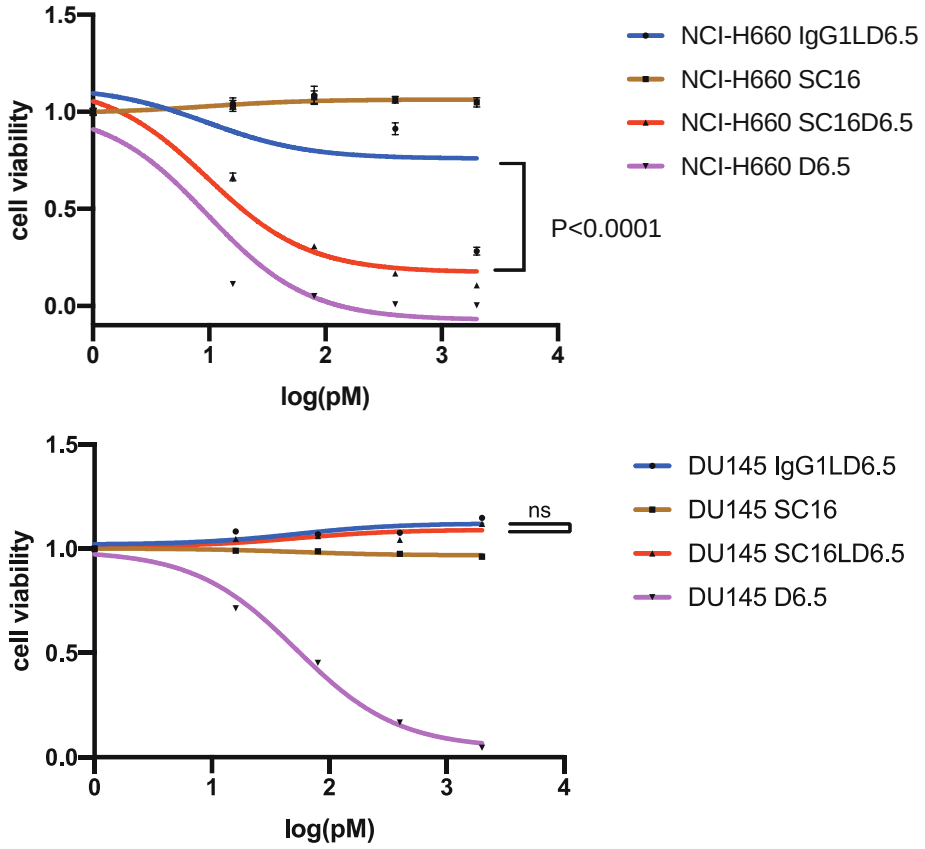

Fig. S16. In vitro toxicity of SC16LD6.5 in NCI-H660 and DU145. Cell viability assay to test SC16LD6.5 (red curve), IgG1LD6.5 (blue curve), SC16 (naked DLL3 antibody) (brown curve), and D6.5 (small molecule PBD warhead) (purple curve) in DU145 and NCI-H660 cell lines. Escalated doses were used as shown on the x axis. For DU145, treatment was performed for 72 hours ($n = 9$ for each treatment dose, error bars: SEM) For NCI-H660, treatment was performed for 13 days with replenishing of drug after 6 days (n=9 for each treatment dose, error bars: SEM). Two-way ANOVA test was used; $p < 0.0001$ for SC16LD6.5 versus IgG1LD6.5 in NCI-H660; not significant (ns) in DU145.

Table S1. Clinical and pathological features of patients with CRPC-Adeno and PCA-expressing DLL3 by IHC.

Table of CRPC-Adeno and localized PCA patients expressing DLL3 with ≥1% of positive cells (intensity 1-2-3) showing SYP expression by IHC, presence of neuroendocrine markers in serum, and pathological features of high grade/ poorly differentiated tumors.

Patient ID	Diagnosis	% DLL3- cells (no expression 0)	% DLL3+ cells (intensity 1)	% DLL3+ cells (intensity 2)	% DLL3+ cells (intensity 3)	SYP (IHC)	Site of mets	High serum CGA and/or NSE	Pathological features
1	CRPC-Adeno	20	70	10	0	n/a	n/a	n/a	High grade adeno carcinoma with histological neuroendocrine features
2	CRPC-Adeno	0	100	0	0	yes	n/a	n/a	High grade adeno carcinoma
3	CRPC-Adeno	0	100	0	0	yes	bone, lymph node, stomach, bladder	n/a	Poorly differentiated carcinoma
4	CRPC-Adeno	0	100	0	0	yes	bone	n/a	Poorly differentiated adeno carcinoma
5	CRPC-Adeno	0	100	0	0	yes	n/a	n/a	High grade adeno carcinoma
6	Localized PCA	98	0	2	0	n/a	bone	yes	Localized adeno carcinoma
7	CRPC-Adeno	99	0	1	0	n/a	bone	yes	High grade adeno carcinoma

Table S2. Genomic features of WCM1388 patient metastatic sites.

Table representing genomic features of prostate and liver tumor from the same patient (WCM1388). *RB1, TP53,* and *PTEN* alterations are reported.

Gene	Prostate	Liver
RB1	deletion	deletion
TP53	mutation	mutation
PTEN	deletion	deletion

Table S3. Clinical features of patients with CRPC-Adeno with DLL3-positive CTCs.

Table presenting features of 5 CRPC-Adeno patients expressing DLL3 in CTCs. Sites of metastasis, neuroendocrine markers in serum, previous therapy, and genomic features (*TP53* and *RB1* loss or mutated) are indicated. Patient ID is indicated as Weill Cornell Medicine (WCM) with an identifying number (patient not treated at WCM is indicated as "no WCM").

ID pts	Site of mets	Serum NE markers level (CGA, NSE)	Platinum-based therapy (yes/no)	Genomic features
WCM758	Liver, bone, lymph node	high	no	No alt
WCM861	Liver, bone, lymph node, lung	normal	no	*TP53* and *RB1* mut
WCM1344	Liver, bone, soft tissue	normal	yes	*TP53* and *RB1* loss
no WCM	Liver, bone, soft tissue, pleural	high	yes	Not available
WCMC13	Liver, lymph node	Not available	yes	Not available

Table S4. Serum pharmacokinetics parameters after peritoneal administration of rovalpituzumab tesirine (SC16LD6.5).

Table showing the pharmacokinetics of SC16LD6.5 (ADC) in NU/J and NOD/SCID mice after intraperitoneal administration of the compound. Serum was collected at 5 min (NOD/SCID only), 4, 24, 72, 168, and 336 hours. Pharmacokinetic parameters (Cmax-maximum serum concentration and exposure [AUC (area under the curve) (0-∞)] are listed in the table. All values summarized by geometric mean.

Strain	Dose [mg/kg]	ADC	
		AUC $_{(0-\infty)}$ [day*µg/mL]	C_{max} [µg/mL]
NU/J	0.25	7.1	1.0
NU/J	0.5	13	1.4
NU/J	1	22	4.3
NOD/SCID	0.2	2.1	1.0
NOD/SCID	0.4	5.8	4.3
NOD/SCID	0.8	12.2	14.1
NOD/SCID	1.6	20.3	33.9