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### ► To cite this version:

Charly Blanc, Anissa Moktefi, Ariane Jolly, Pierre De La Grange, Denise Gay, et al.. The Neuropilin-1/PKC Axis Promotes Neuroendocrine Differentiation and Drug Resistance of Prostate Cancer. *British Journal of Cancer*, 2023, 15, 10.1038/s41416-022-02114-9 . hal-03998379

**HAL Id: hal-03998379**

**<https://hal.u-pec.fr/hal-03998379>**

Submitted on 15 Dec 2023

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# The Neuropilin-1/PKC Axis Promotes Neuroendocrine Differentiation and Drug Resistance of Prostate Cancer

**Running title:** NRP1 drives Prostate cancer multi-resistance through PKC activation.

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**ABSTRACT**

**Background:** Neuroendocrine prostate cancer (NEPC) is a multi-resistant variant of prostate cancer (PCa) that has become a major challenge in the clinics. Understanding neuroendocrine differentiation (NED) process at the molecular level is therefore critical to define therapeutic strategies that can prevent multi-drug resistance.

**Methods:** Using RNA expression profiling and immunohistochemistry, we have identified and characterized a gene expression signature associated with the emergence of NED in a large PCa cohort including 169 hormone-naïve PCa (HNPC) and 48 castration-resistance PCa (CRPC) patients. *In vitro* and preclinical *in vivo* NED models were used to explore the cellular mechanism and to characterize the effects of castration on PCa progression.

**Results:** We show for the first time that Neuropilin-1 (NRP1) is a key component of NED in PCa cells. NRP1 is upregulated in response to androgen deprivation therapies (ADT) and elicits cell survival through induction of the PKC pathway. Downmodulation of either NRP1 protein expression or PKC activation suppresses NED, prevents tumor evolution toward castration resistance and increases the efficacy of docetaxel-based chemotherapy in preclinical models *in vivo*.

**Conclusions:** This study reveals the NRP1/PKC axis as a promising therapeutic target for the prevention of neuroendocrine castration-resistant variants of PCa and indicates NRP1 as an early transitional biomarker.

**Key words:** Neuroendocrine Differentiation, Prostate Cancer, Therapeutic Resistance, Neuropilin-1 (NRP1), PKCs signaling.

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## BACKGROUND

The mortality associated with prostate cancer (PCa) is mainly due to its progression to therapeutic-resistant metastatic disease. Androgen deprivation therapies (ADT) have improved the management of the disease however the vast majority of tumors ultimately acquire resistance to ADT. In most cases, this is associated with genomic alterations affecting the androgen receptor (AR) axis and apoptotic pathways (1). Thus, reactivation of AR signaling contributes to tumor cell survival, proliferation, metastatic spread, and to the development of castration-resistant prostate cancer (CRPC). However, other mechanisms have also been implicated in the development of CRPC as illustrated by the emergence of “non-AR-driven” neuroendocrine prostate adenocarcinoma (NEPC) (2).

Over a decade, a number of approved and promising therapies for CRPC have emerged, including taxane chemotherapies and AR pathway inhibitor strategies such as enzalutamide (3-5) and apalutamide, a next-generation AR inhibitor (6). Compelling evidence suggests that prolonged treatment induces lineage crisis, associated with the progression of drug-resistant CRPC leading to PCa-related death (7). Tumor cell acquisition of a neuroendocrine phenotype (NE) has been linked to drug resistance. Typically, NE differentiation (NED) is distinguished by reduced AR expression or activity as well as low expression of androgen-regulated genes (including PSA) and upregulation of NE markers (8, 9). Recent studies have demonstrated that NEPC can be associated with recurrent genetic lesions including loss of tumor suppressors, such as *RBI* (10) and p53 (10, 11), overexpression and genomic amplification of *MYCN* and *AURKA* (12, 13), and deregulation of epigenetic regulators/mediators such as BRN2 (14), REST (15) and EZH2 (12, 13), suggesting a late stage involvement. However, the work of others has shown that at least several of these key genes are upregulated early and may have roles in tumor cell transition to drug resistance (16).

We hypothesized that by examining the expression of a neuronal genes panel (Neurogenesis GO:0022008), we could identify drivers and potential therapeutic targets of NED and drug resistance. In comparing expression profiles of PCa tumors in cohorts comprising HNPC (54 patients) and CRPC (13 patients) phenotypes, we identified 92 neurogenesis genes within the CRPC cohort, several of which correlate with the NE PCa phenotype. Characterization of this gene set identified the transmembrane glycoprotein Neuropilin-1 (NRP1).

1 NRP1 has been identified as an androgen-repressed gene whose expression is up-regulated during the  
2 adaptive response to ADT (16). Functional studies by this group revealed that NRP1 is likely involved in PCa  
3 metastatic migration via upregulation of EMT genes. However, unrelated work has demonstrated that NRP1  
4 has additional functions in development and potentially tumorigenesis (17) (18) , some of which require PKC  
5 and AKT signaling upregulation (19).

6 In this report, we confirm that NRP1 is upregulated in transition to ADT and further, our examination  
7 of human PCa datasets suggests that it may be present in 28% NEPC tumors. Importantly, we show that NRP1  
8 is requisite for ADT transition in *in vitro* studies. Mechanistically, NRP1 induces expression and activation of  
9 the PKC pathway leading ultimately to increased cell survival. Finally, specific inhibition of the PKC pathway  
10 sensitizes PCa cells to chemo-hormonal treatment. Together, our findings provide crucial insights into a novel  
11 NRP1/PKC axis to reveal promising new therapeutic targets in the treatment of PCa patients with NED and  
12 point to NRP1 as an early biomarker in tumor cell transition to the drug resistant NE phenotype.

## 1 **MATERIALS AND METHODS**

2 The source and catalog number of primary antibodies (Ab) are listed in Table S1. The experimental methods  
3 not described herein are provided in Supplementary Data.

### 4 5 **Human Prostate Cancer Specimens**

6 Prostate tissue samples were collected as part of an Institutional Review Board approved protocol at Henri  
7 Mondor Hospital in France. This study included 169 PCa patient samples (from radical prostatectomy) without  
8 having received prior hormone treatment at the institution (HNPC) and 48 CRPC tumors (collected by  
9 transurethral resection). CRPC tumors were separated in 27 CRPC-Adeno with less than 20% of  
10 neuroendocrine differentiation and 21 CRPC-NE with more than 20% of neuroendocrine differentiation as  
11 described (2, 8). Immunohistochemistry of synaptophysin and chromogranin-A as NE markers were  
12 performed to attribute the percentage of NED. The study also included a few specimens derived from normal  
13 prostates from peritumoral tissues. Demographic, clinical, and pathological parameters were collected  
14 prospectively in a database and retrospectively reviewed. Tumors were classified by the following criteria  
15 based on histomorphology by genitourinary pathologist (Y. Allory).

### 16 17 **RNA Microarray and Transcriptomic Data**

18 Total RNA was isolated from frozen tissues using the miRNeasy kit (Qiagen) and transcriptome profiles were  
19 generated from HNPC (n = 54) and CRPC (n = 13) prostate cancer tissues and analyzed using GeneChip®  
20 Human Transcriptome array 2.0 (ThermoFisher Scientific). Data have been analyzed by Genosplice company  
21 (P. de la G range and A. Jolly) as previously described (20, 21).

### 22 23 24 **Immunohistochemistry**

25 Immunohistochemistry (IHC) studies were performed as previously described (22) on formalin-fixed paraffin-  
26 embedded (FFPE) of all tissue samples. All slides were read by a genitourinary pathologist (A. Moktefi). For  
27 NRP1 staining analysis, numerical score was assigned as no staining (0), low staining (1), moderate staining

(2) and strong staining (3). Staining was considered positive when numerical score was  $\geq 2$ , because normal glands are weak or negative.

#### **Cell Culture**

PCa cell lines (VCaP, LNCaP, 22Rv1, PC3 and DU145) were obtained from American Type Culture Collection and grown in RPMI-1640 (22Rv1, PC3, DU145, C4-2), DMEM (VCaP), or DMEM/RPMI (LNCaP) supplemented with 10% fetal bovine serum (FBS) (ThermoFischer Scientific, France). LNCaP-NE cells were obtained from LNCaP cells cultured in androgen-reduced condition (phenol red-free DMEM/RPMI supplemented with 10% charcoal-stripped serum (CSS)).

Overexpression of NRP1 was obtained by stably transfecting LNCaP, C4-2 and 22Rv1 cell lines with the pCherry-mNrp1 plasmid (Addgene plasmid # 21934; **(23)**) using lipofectamine 2000 (Invitrogen) standard protocol. Cells selection was performed in medium containing G418 (300  $\mu\text{g/ml}$  for C4-2 cells and 400  $\mu\text{g/ml}$  for LNCaP and 22Rv1).

#### **Subcellular Fractionation, Western Blot and Immunoprecipitation**

Cells were washed with cold PBS and lysed in 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease and phosphatase inhibitors cocktail (Roche). For cytosol and membrane proteins extractions, cells were prepared using a subcellular fractionation kit (Thermo Scientific) with protease and phosphatase inhibitors according to the manufacturer's instructions.

For immunoprecipitation, proteins were prepared using lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10% glycerol, and protease and phosphatase inhibitors. 500  $\mu\text{g}$  of total protein extract was incubated with 1  $\mu\text{g}$  of anti-NRP1 antibody or control IgG overnight at 4°C. Complexes were pulled-down using Bio-Adembeads Protein A/G magnetic beads (Ademtech), washed with lysis buffer and analyzed by SDS-PAGE. Immunostaining were visualized using the GBox system (Syngene). Band intensities were quantified using the Multi Gauge v3.0 software (Fujifilm).

#### **Small Interference RNA Assay**

1 siRNA transfections for LNCaP, C4-2 and PC3 cells were performed using Lipofectamine RNaiMax  
2 (Invitrogen) according to the manufacturer's protocol. Experimental conditions were optimized for LNCaP-  
3 NE. Briefly, LNCaP-NE cells were seeded in Poly-L-Lysine (Sigma) coated 12-wells at 70% confluence and  
4 then transfected with 200 pmol of either non targeting siRNA 5'-GGUGCGCUCCUGGACGUAGCC-3' as a  
5 control or target-specific NRP1 5'-GGCUACGUCCAGGAGCGCACC-3' or PKC isoforms siRNAs with  
6 Lipofectamine Messenger MAX (Invitrogen). PKC $\alpha$ , PKC $\epsilon$  and PKC $\delta$  - siRNA were a gift from Dr K. Mahéo  
7 (Inserm UMR 1069, Tours, France) and are referenced (24). Transfection efficiency was evaluated by Western  
8 blot analysis.

### 10 **Xenograft Studies**

11 All mouse experiments were performed according to guidelines on animal care and with appropriate  
12 institutional certification of ethical comity and conducted in compliance with European Community. LNCaP  
13 ( $2 \times 10^6$  in 50% Matrigel) or PC3 ( $2 \times 10^6$ ) cells were injected subcutaneously into the right flank of 5-week-  
14 old male NMRI nude mice (Janvier, Le Genest-Saint-Isle, France). In the Enzastaurin/castration combination  
15 experiment, mice bearing LNCaP tumors of about 200-300 mm<sup>3</sup> were separated randomly in several groups.  
16 Mice were then castrated by bilateral orchiectomy and treated one day after castration with 100 mg/kg  
17 Enzastaurin or vehicle by oral gavage every day. In the Enzastaurin/docetaxel combination experiment, mice  
18 bearing PC3 tumors of about 50-80 mm<sup>3</sup> were separated randomly in several groups and treated with 100  
19 mg/kg Enzastaurin or vehicle by oral gavage every day and/or with 5 mg/kg docetaxel at or PBS vehicle by  
20 intraperitoneal injection once a week. Tumor size was measured two times per week with a caliper and tumor  
21 volume was calculated with the formula:  $V = 4/3\pi \cdot R1^2 \cdot R2$  whereby radius 1 (R1), radius 2 (R2). Then,  
22 percentage of tumor size was assigned to 100% at the beginning and each measure represents the percentage  
23 of tumor growth evolution.

### 25 **Analyses of single cell RNAseq datasets.**

26 ScRNAseq analysis of primary prostate tumors (25) was undertaken using the publicly available web tool at  
27 [www.pradcellatlas.com](http://www.pradcellatlas.com). The epithelial atlas was explored in this work.



1 To analyze and compare results with publicly available scRNAseq data from a castration-resistant LNCaP cell  
2 model (26), datasets from GSE205765 were downloaded from GEO, transformed into Seurat objects, MT  
3 levels set at 10% for each object and objects integrated using Seurat pipelines (26). Clusters exhibiting high  
4 mitochondrial or ribosomal signatures were considered non-viable and removed from the analysis. The Find  
5 Clusters resolution parameter was set low to generate clusters with only fundamental transcriptional  
6 differences. Cluster (c)0 included both FCS and CSS cells (33%, 52% respectively), c1 was almost exclusively  
7 FCS (60%, 1% respectively), c2 almost exclusively CSS (7%, 47%) as previously shown. Dotplot analysis  
8 uses a Seurat tool.

9

### 10 **Statistical Analysis**

11 Pearson correlations were implemented for gene-gene expression correlation using GraphPad Prism  
12 (GraphPad Software). In bar graphs and dose-response curves, comparisons between each group were  
13 performed using Student's *t* test or multiple *t* test. All statistical tests used a two-tailed  $\alpha = 0.05$  level of  
14 significance and were performed using GraphPad Prism (GraphPad Software). For *in vitro* studies, graphs  
15 show pooled data with error bars representing  $\pm$  SEM obtained from at least three independent experiments.  
16 Statistical significance was accepted for \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

17

18

## 1 RESULTS

### 2 Validation of the Mondor Patient dataset and identification of a neuronal transcriptional program 3 within the CRPC subset.

4 A neuroendocrine (NE) phenotype correlates with aggressive treatment-resistant tumors in treatment-resistant  
5 prostate cancer. Rigorous sets of signature genes have been identified, including CHGA, SYP, TUBB3, EZH2  
6 ((27), (28)). To identify potential novel candidates involved in transition from a CRPC-adenocarcinoma to  
7 NE phenotype in ADT resistant patients' tumors, whole-transcriptome profiles were analyzed using  
8 oligonucleotide microarrays from 13 CRPC and 54 localized HNPC tumors (Mondor Dataset). Clinical  
9 characteristics are summarized in **Table S2**. Comparative analysis between CRPC and HNPC showed a  
10 significant differential expression in 1,849 genes (Heatmap, **Fig. 1A** and **Table S3**). Examination of CRPC  
11 upregulated genes, using the functional annotation tool DAVID, showed that many were involved in cell cycle,  
12 microtubule alterations, negative regulation of apoptosis as expected, as well as serine/threonine protein kinase  
13 and neuronal signatures (**Fig. 1B**).

14 Examination of the Mondor dataset for AR-regulated genes using a signature previously defined by  
15 Hieronymus et al., 2006 (29) (see **Table S4**) revealed an overall lower expression in CRPC as compared to  
16 HNPC (p=0.00042; **Fig. 1C Left**). This result was confirmed by immunohistochemistry (IHC) with lower  
17 protein expression of androgen responsive gene PSA in CRPC compared to HNPC tissues (two representative  
18 samples, **Fig. 1C Right**, and data not shown). Concomitant with the decrease in AR targeted genes, we  
19 observed a modest increase in the CRPC NE profile using an NEPC signature ((2), see **Table S5**) (**Fig 1D**).

20 We hypothesized that some overlapping members between a defined neurogenesis signature (GO:0022008)  
21 and the Mondor dataset might provide candidates for PCa transition to an NE phenotype. Examination of the  
22 Mondor dataset against this signature revealed upregulated expression of *NRP1*, *NRP2*, *AURKA*, *EZH2*,  
23 *LAMB1*, *NLGN1* genes (**Fig 1E, Table S6**), several of which have already been identified in the NE phenotype  
24 (*AURKA*, *EZH2*, *NLGN1*).

25

26 **NRP1 is an EARLY INDUCTION GENE FOR NED.**

1 We decided to focus on NRP1 in NED because it has been linked to high Gleason score and ADT (16) and  
2 functionally linked to regulation of PKC and AKT pathways (19), (30) (31), both observed in the DAVID  
3 analysis (Fig1B).

4 To validate it as a potential candidate, we observed that NRP1 expression was upregulated in the CRPC  
5 Mondor dataset (Fig 1F, Fig S1A), downregulated against AR genes within the Mondor dataset (Fig S1B)  
6 and showed increasing protein expression from HNPC to CRPC-NE stages (Fig 1G, Fig S1C).

7 To further examine timing and location of NRP1 in PCa, we relied upon a publicly available single cell  
8 RNAseq (scRNAseq) analysis of 13 tumor biopsies from 12 PCa patients, including those with luminal, basal,  
9 or proliferative phenotypes (25) for details). Fig S2A shows an integrated UMAP of clusters based on gene  
10 similarity: cluster 10 represents basal, cluster 12 proliferative and all other clusters luminal epithelial  
11 phenotypes. The authors categorized basal and luminal clusters as mainly non-malignant/unresolved and  
12 malignant/unresolved, respectively. Using their on-line interactive tool to investigate genes of interest  
13 ([www.pradcellatlas.com](http://www.pradcellatlas.com), Epithelial dataset), we found that NRP1 and other neuronal genes from the  
14 neurogenesis signature comparison (Fig 1E) were similarly expressed across luminal clusters in most patients  
15 and absent from either basal or proliferative clusters. Interestingly, most were absent from clusters 2 and 7,  
16 derived primarily from a single patient, which express high levels of *AR* and *KLK3*. These results confirm that  
17 NRP1 is expressed even before post-operative treatment.

18 To ask about NRP1 expression after ADT, we examined the SU2C-PCF dataset (208 CRPC mRNA samples  
19 across both Adeno-CRPC and NEPC (11%), (32). Clusterplots show NRP1 expression was upregulated in  
20 approximately 12% samples (Fig S2C). Co-expression of NRP1 and different NE signature genes revealed  
21 that although overall co-expression against *CHGA*, *SYP*, *TUB33*, *EZH2*, *TP53* showed low to negative  
22 Spearman scores, other NE genes correlated positively across the entire cohort and most co-expression  
23 analyses revealed at least some samples with positive correlation (Fig S2C).

24 To better focus on NE samples only, a cohort of 39 patients (37 mRNA samples) with high NEPC and low  
25 AR scores (see Table 7) was selected from the SU2C-PCF dataset. Of the 37 samples, 8 expressed NRP1  
26 mRNA (Fig S3).

27 To try to define a true NEPC population, we examined expression of *CHGA*, *SYP* and *EZH2*, all NE signature  
28 genes, and found 14 samples positive for all 3 genes. Of these 14, 4 samples also expressed NRP1 (Fig S3).

1 These combined results confirm upregulated expression of NRP1 in 12% CRPC samples overall, increased  
2 (22%) within a selected NEPC-High cohort. Further, within our putative NEPC population, approximately  
3 29% co-expressed NRP1.

4 We then examined NRP1 expression in the LNCaP NED model, in which LNCaP-NE cells emerge from long-  
5 term culture of LNCaP cells in androgen-deprived medium (33, 34).

6 As expected, NRP1 protein levels were low in epithelial cell lines LNCaP, VCaP C4-2, 22RV1 and DU145  
7 and moderate to high in cell lines displaying a pronounced NE phenotype such as LNCaP-NE, C4-2-NE, or  
8 small cell NE-like PC3 cells (Fig. S4A). LNCaP-NE cells were validated by morphological changes, down-  
9 regulation of androgen-regulated genes (*KLK3/PSA*) and up-regulation of NE markers such as CHGA, neuron  
10 specific Enolase (NSE) and  $\beta$ -Tubulin III (Fig. 2A). Interestingly, basal NRP1 protein levels rose very early  
11 in the time course and remained elevated over time (Fig. 2A).

12 As previously observed (16), we found that NRP1 expression is negatively regulated by the AR pathway.  
13 Treatment of LNCaP-NE and C4-2-NE cells with dihydrotestosterone (DHT) strongly reduced expression of  
14 both NRP1 and NE markers and increased the androgen-regulated protein PSA (Fig. 2B, C and Fig. S4B).  
15 Inversely, knockdown of AR in LNCaP or C4-2 cells increased NRP1 expression, as well as NE marker  $\beta$ -  
16 Tubulin III, compared to the non-targeting siRNA control (Fig. 2D and Fig. S4C). Similar results were  
17 observed upon treatment of cells with the AR inhibitor enzalutamide (Fig. 2E and Fig. S4D). Importantly, AR  
18 impacts NRP1 promoter activity since this activity was increased by enzalutamide or androgen-depleted  
19 condition and decreased by treatment with DHT (Fig. 2F).

20 To ask if NRP1 expression might be required for the NE phenotype, we stably transfected LNCaP cells with  
21 an NRP1-expressing vector. Western blot analysis revealed that these cells displayed a neuronal morphology  
22 with upregulation of NE markers, and down-regulation of androgen-regulated protein PSA (Fig. 2G).  
23 Conversely, knockdown of NRP1 expression using siRNAs diminished NE marker expression in LNCaP-NE  
24 cells (Fig. 2H). Exogenous expression of NRP1 in 22Rv1 and C4-2 cells, further confirmed this link (Fig. S4,  
25 E and F). These results demonstrate for the first time that NRP1 expression is directly associated with the  
26 NED process and may be an early requisite for transition.

27  
28 **NRP1 drives NED through the PKC signaling pathway.**

1 NED-inducing stimuli have been shown to increase intracellular levels of cAMP for activation of the  
2 transcription factor cAMP response element-binding protein (CREB) (35, 36). We explored phosphorylation  
3 pattern differences in LNCaP and LNCaP-NE cells using a CREB pathway phospho-antibody array containing  
4 174 antibodies. Overall, more proteins were found to be phosphorylated in LNCaP-NE than in LNCaP cells  
5 (Fig. 3A) and several key components of CREB, AKT and ERK pathways were phosphorylated in NED (Fig.  
6 3A), as previously reported (37).

7 The phospho-specific protein microarray analysis also indicated increased phosphorylation within the PKC  
8 pathway in LNCaP-NE cells compared to LNCaP cells (Fig. 3A). We examined this pathway in greater detail  
9 because NRP1 function has been linked to PKC activation (38). DAVID analyses confirmed the importance  
10 of Ser/Thr pathways and *PRKCD* was identified in the Mondor CRPC upregulated genes list (Fig 1B, Table  
11 S3). We confirmed increased expression of PKC and increased phosphorylation at PKC pan-activation site  
12 Ser660 by Western blot analysis following a time course of androgen depletion in LNCaP cells (Fig. 3B).  
13 NRP1 overexpression in LNCaP, C4-2 or 22Rv1 cells also resulted in increased PKC phosphorylation  
14 compared to control vector-transfected cells (Fig. 3C), suggesting that PKC activation might be NRP1-  
15 dependent. In support of this, a variety of downstream PKC targets were examined during transition from  
16 LNCaP to LNCaP-NE phenotype and in stably transfected 22Rv1 cells overexpressing NRP1 (Fig S5). Both  
17 ERK and Akt exhibited increased phosphorylation and notably, apoptosis inhibitor Bcl2 was upregulated (Fig  
18 S5).

19 PKCs form a large family including the widely characterized isoenzymes (PKC $\alpha$ , PKC $\beta$ , PKC $\gamma$ , PKC $\delta$ , PKC $\epsilon$ )  
20 expressed in multiple cancers and during neuronal differentiation (39). Examination of the Mondor dataset  
21 showed that transcription of *PRKCA* and *PRKCD* were significantly upregulated in CRPC compared to HNPC  
22 tumors (Fig. S6A). Examination of a scRNAseq dataset comparing hormone-intact (FCS) and castrate-  
23 condition (CSS) LNCaP cells (26), GSE205765, Fig. S6B) confirmed upregulation of *PRKCA* and *PRKCD* in  
24 CSS clusters only (See Materials and Methods for details). Upregulated phosphorylation was also observed in  
25 PKC $\alpha$ , PKC $\delta$  and PKC $\epsilon$  proteins from LNCaP-NE compared to LNCaP cells (Fig. S6C). Finally, because  
26 PKC is activated at the plasma membrane, we examined distribution of NRP1 and isoforms PKC $\delta$  and PKC $\alpha$   
27 in cytosol and membrane fractions of LNCaP and LNCaP-NE cells. High levels of EGFR or GAPDH in  
28 membrane or cytosol fractions, respectively, showed the fraction purity (Fig. S6D Left). As expected, NRP1

1 was upregulated in LNCaP-NE cells and primarily localized to the cell membrane. Expression of both PKC $\delta$   
2 and PKC $\alpha$  isoforms was also upregulated in LNCaP-NE cells and primarily localized to the cell membrane  
3 (**Fig. S6D** Right).

4 We next examined the relationship between NRP1 and PKC isotypes in NED. Cross correlation of NRP1 and  
5 PKCD in the Mondor dataset suggested that increased transcription was tightly coupled (**Fig 3D**). Further,  
6 NRP1 transcriptional silencing in PC3 cells, which strongly expressed this protein, resulted in a significant  
7 reduction in PKC $\delta$  protein levels but did not change PKC $\alpha$  and PKC $\epsilon$  levels (**Fig. 3E**). Finally, co-  
8 immunoprecipitation experiments revealed that NRP1 capture resulted in co-IP of PKC $\delta$  in LNCaP-NE and  
9 PC3 cells (**Fig. 3F**). Although NRP1 activation of the PKC pathway in endothelial cells requires VEGF and  
10 VEGFR co-receptors (**38**), examination of the GSE205765 scRNAseq dataset found none of these mediators  
11 in either FCS or CSS condition (data not shown). Taken together, these results indicate that NRP1 positively  
12 regulates PKC expression and activation in NED and may directly associate with specific isoforms in the cell  
13 membrane.

14 To ask which PKC isoform(s) might be requisite for the NE phenotype, siRNAs were used to target PKC $\alpha$ ,  
15 PKC $\delta$  or PKC $\epsilon$  in LNCaP-NE cells and NE profile markers examined by western blot. Knockdown of PKC $\delta$   
16 resulted in a significant reduction of CHGA and SYP, suggesting a reversal of the NE phenotype (**Fig. 3G**).

17 We also observed co-expression of PKC $\alpha$  and PKC $\delta$  and downstream target BCL2 in our SU2C/PFC NEPC  
18 NRP1+ population (**Fig. S3**), confirming existence of this phenotype in human advanced PCa samples.

19 Together, these results suggest that PKC expression is linked to NRP1 and that PKC might be a significant  
20 contributor to the NE phenotype.

### 22 **Inhibition of the PKC pathway counteracts NED and blocks CRPC progression *in vivo*.**

23 Based on the findings that PKC expression and activation are upregulated early in NED and could drive NE  
24 transdifferentiation (**Fig. 3**), we postulated that treatment with the PKC inhibitor Enzastaurin would reverse  
25 NED. Although initially described as a PKC $\beta$  inhibitor, enzastaurin has broad impact on other PKC isoforms,  
26 including PKC $\alpha$  and PKC $\delta$ , and is frequently used as a pan-PKC inhibitor (**40**), (**41**), (**42**). Treatment of  
27 LNCaP-NE cells with Enzastaurin resulted in decreased phosphorylation of pan-PKC and significantly  
28 reduced expression of NE markers (**Fig. 4A**). LNCaP cells stably overexpressing NRP1 (LNCaP-NRP1)

1 exhibited reduced viability in the presence of Enzastaurin compared to control LNCaP cells with a decreasing  
2 GI<sub>50</sub> from 8.9 to 5.3  $\mu$ M (**Fig. 4B**). SiRNA knockdown of PKC isoforms in LNCaP-NE cells confirmed their  
3 diminished viability of through PKC activity (**Fig. 4C**).

4 We then evaluated the combined effect of castration and Enzastaurin on tumor growth *in vivo* using LNCaP  
5 ectopic xenograft model. The LNCaP model is commonly used *in vitro* and *in vivo* to model the response to  
6 ADT of PCa. Xenografted male mice were castrated by surgery to block androgen synthesis and promote an  
7 apparent LNCaP-NE phenotype as defined by drug resistance in injected LNCaP tumor. In response to  
8 castration, tumor growth in control mice remained low for two weeks, after which time tumor cells proliferated  
9 despite castration, and developed androgen-independent tumors (**Fig. 4D**). Enzastaurin treatment resulted in  
10 a significant inhibition of tumor growth over 30 days post-castration (**Fig. 4D**). Taken together, these and  
11 above results support the implication of a NRP1/PKC axis in CRPC that promotes the survival of NE cells.  
12 They further indicate that Enzastaurin treatment can counter PCa progression.

#### 14 **The NRP1/PKC axis confers resistance to taxane chemotherapy.**

15 We have previously reported that CRPC cells with upregulated NE phenotype are resistant to a wide range of  
16 cytotoxic agents, including taxane chemotherapies (**34**). Moreover, analysis of RNA-sequencing data of 150  
17 metastatic CRPC bone or soft tumor biopsies from the Robinson cohort (**43**) showed a trend toward increased  
18 expression of NRP1 in patients treated with taxane chemotherapies (**Fig. 5A**). *In vitro*, docetaxel treatment of  
19 LNCaP cells induced the expression of NRP1 in cells that survive following treatment (**Fig. 5A**). These results  
20 strongly suggest a role for NRP1-induced NED in resistance to docetaxel. To answer this question, LNCaP-  
21 NRP1 and LNCaP-vector cells were treated for 72 h with docetaxel and cell viability was measured by MTT.  
22 NRP1 overexpression resulted in a lower sensitivity to docetaxel with an increase of GI<sub>50</sub> from 1 to 4nM (**Fig.**  
23 **5B**). Similar results were obtained by overexpressing NRP1 in 22Rv1 and C4-2 cell lines (**Fig. S7A and S7B**).  
24 In addition, NRP1 overexpression in LNCaP cells conferred resistance to docetaxel-induced apoptosis in a  
25 dose-dependent manner (**Fig. 5C**). Notably, NRP1 overexpression in LNCaP cells also increased the  
26 expression of Bcl-2,  $\beta$ -Tubulin III and MDR-1 in LNCaP-NRP1 compared with LNCaP-vector cells (**Fig.**  
27 **S7C**), all known to be implicated in cell survival and taxane resistance (**44**) (**45**).

1 The PKC pathway has also been shown to play a role in chemotherapeutic resistance (46). Enzastaurin  
2 treatment significantly enhanced the antitumoral effect of docetaxel on LNCaP-NE and metastatic, castration-  
3 resistant PC3 cells (Fig. 5, D and E). PC3 cells are documented to have an NE phenotype and have been used  
4 in this context by others (47) (48). In *in vivo* studies, a combination of Enzastaurin with docetaxel resulted in  
5 a stronger inhibitory effect on xenografted PC3 tumor growth when compared with single-agent treatments  
6 (Fig. 5F). These results indicate that Enzastaurin treatment may counteract taxane-resistance and potentially  
7 enhance the response to docetaxel in CRPC-NE.

## 9 DISCUSSION

10 Our results showed that NRP1, a transmembrane glycoprotein expressed in a wide variety of human cancers,  
11 and required for aggressive tumor growth and tumor-related angiogenesis (reviewed in (49), is an important  
12 early player in PCa drug resistance and a putative candidate for its induction. Our analyses of expression  
13 profiles from human PCa specimens (Mondor dataset) identified NRP1 as a central feature amongst the CRPC  
14 cohort overexpressed NE-related genes. We found that NRP1 was also detectable in HTPC-Adeno and CRPC-  
15 Adeno specimens (data not shown) and primary luminal tumor samples (25), indicating that low NRP1  
16 expression precedes transition to drug resistance. In agreement with others (16), we also observed that  
17 androgens maintain NRP1 at low levels and that release from their effects promotes strong upregulation of  
18 both NRP1 transcription and protein expression. Importantly, our NRP1 overexpression and knockdown  
19 studies showed for the first time that NRP1 may be a key player in transition to therapy resistance. Its  
20 upregulation during ADT transition and potential role(s) in the induction of ADT-NE indicate NRP1 as a  
21 novel biomarker and a target for more efficacious therapy to prevent PCa drug resistance.

22 Our examination of the human SU2C-PCF CRPC dataset revealed that NRP1 and downstream PKC players  
23 are expressed in a subset of NEPC tumors. Others have observed plasticity in advanced PCa associated with  
24 epigenetic reprogramming driven by N-Myc (50). Thus, multiple NEPC subtypes likely exist reflecting this  
25 (and potentially other unrelated) epigenetic transitions. We postulate that, based upon early expression of  
26 NRP1 in NE transformation, it will play a seminal role in NE transformation upstream of further alterations.  
27 Because of its limited molecular characterization, there is no standard treatment for patients with NEPC.  
28 Although multiple pathways, including PI3K/AKT and MAPK, likely converge to drive the emergence of NE



1 phenotype, no studies so far have demonstrated clear clinical benefits from targeting these pathways (51). In  
2 our CRPC-NE models, inhibition of these pathways with specific inhibitors (PI3K inhibitor LY294002 and  
3 ERK inhibitor PD0325901) have failed to reverse NE phenotype (data not shown), suggesting that these  
4 pathways may not have direct impact in NRP1-induced NED.

5 Our results show that NRP1 upregulation correlates with PKC activation as determined by increased  
6 phosphorylation during ADT and that NRP1 co-immunoprecipitates with PKC, suggesting a functional link.  
7 Further, both NRP1 overexpression and knockdown studies support the notion that NRP1 upregulation during  
8 ADT is directly required for PKC activation. Work in unrelated fields has shown that NRP1 directly associates  
9 with VEGFRs for VEGF-mediated PKC induction during angiogenesis (38) We have not identified either  
10 VEGFRs or VEGF in our cultures (data not shown), but NRP1 is a well recognized pleiotropic receptor and  
11 has been shown to associate with plexins, PDGFRs, leptin, etc. to mediate numerous downstream functions  
12 (52), (53). Identification of specific NRP1 co-receptors and ligands for PKC induction in drug-resistant PCa  
13 is a current focus of our lab.

14 Microarray data from the patient cohort (Mondor dataset) showed that PKC $\alpha$  and PKC $\delta$  were most highly  
15 expressed in human CRPC and our *in vitro* results and examination of a scRNAseq database (26) confirmed  
16 this finding. While our *in vitro* data support a clearer role for PKC $\delta$  in NRP1-induced NED, PKC $\alpha$  may also  
17 play a role in cell survival as demonstrated in knockdown studies. These results warrant further studies to  
18 clarify this point.

19 PKC pathway activation in response to androgen deprivation has been shown to promote resistance to AR-  
20 targeted therapy (54). We found that PKC $\alpha$  or PKC $\delta$  knockdown significantly decreased LNCaP-NE survival  
21 *in vitro* and that *in vivo* treatment with enzastaurin, a powerful pan-PKC inhibitor, of castrated mice injected  
22 with LNCaP tumor cells resulted in significant reduction of tumor growth. Both results support a role for PKC  
23 activity in tumor cell viability. Importantly, comparison of control and NRP1-overexpressing LNCaP cells  
24 after enzastaurin treatment revealed that only NRP1-overexpressing cells were susceptible to treatment  
25 resulting in reduced viability, thus further confirming an important role for NRP1 in PKC activation.

26 For decades, taxane-based chemotherapies have been the main treatment for metastatic CRPC. Although it  
27 prolongs overall survival for some patients, many do not respond to treatment, while others invariably develop  
28 resistance. We have previously reported that CRPC-NE cells are resistant to multiple cytotoxic agents (34).

1 In our *in vitro* study, we demonstrated that NRP1 promotes higher resistance to docetaxel-based chemotherapy  
2 concomitantly with the acquisition of NE phenotype.

3 Numerous cellular pathways involving apoptosis, signaling components, drug efflux pumps and tubulin are  
4 implicated in the development of chemoresistance (55). Both NRP1 and the PKC pathway have been  
5 implicated in drug resistance in multiple cancers (46). Here, we have shown that NRP1 over-expression in  
6 LNCaP cells induces the expression of some key players in cell survival and taxane resistance, including Bcl-  
7 2,  $\beta$ -Tubulin III and MDR-1. All these are known PKC downstream targets (56). We also show that  
8 Enzastaurin increases the cytotoxic effects of docetaxel in CRPC-NE cells *in vitro* and in a preclinical model  
9 *in vivo*. Altogether, these findings point to an important NRP1/PKC axis that promotes tumor cell survival  
10 and docetaxel resistance.

## 11 CONCLUSIONS

12 While several aspects of therapy-resistant treatment-induced NEPC have been explored, how to  
13 therapeutically target these aggressive metastatic NE subsets remains a clinical challenge (51). We propose  
14 that PKC inhibitors could be used as novel co-targeted therapies in an adjuvant setting combined with AR-  
15 directed therapy and cytotoxic chemotherapy in the treatment and/or prevention of multi-resistant CRPC-NE  
16 disease. The clinical potential of targeting the PKC pathway with Enzastaurin has been demonstrated in  
17 neuroendocrine pancreatic cancer (57). In the prostate cancer setting however, a phase II trial evaluating  
18 Enzastaurin in combination with docetaxel for patients with PSA progression in CRPC was disappointing in  
19 so far that it showed no benefits in combination (58). Nevertheless, it remains that PSA progression in castrate  
20 state may not be an ideal inclusion criteria because CRPC-NE most likely displays reduced if no expression  
21 of PSA. In such studies, it would be interesting to include patients with a high CRPC-NE contingent.

22 Our work reveals several novel findings with implications for patients with CRPC and drug-resistant NE  
23 disease. These findings support a real promising clinical value of the NRP1/PKC-targeted axis in the treatment  
24 and prevention of therapy-resistant treatment-induced NED. NRP1 would provide an excellent biomarker of  
25 PCa progression and particularly early diagnosis of NE disease.

## 1 **ADDITIONAL INFORMATION**

### 2 **Acknowledgments:**

3 We thank gratefully Patricia Zadigue (INSERM U955, Equipe 7, UPEC), Alexandre Fifre (CRRET  
4 Laboratory, UPEC) for providing advices and technical assistance. We thank Karine Mahéo for her scientific  
5 and technical supports for studying PKC pathway (UMR, INSERM U 1069, Tours). We thank Richard  
6 Souktani and Cécile Lecointe who are in charge of the Small Animal Functional Exploration Platform and  
7 IMRB (INSERM, U955) Pet Shops, Aurélie Guguin and Adeline Henry for the Flow Cytometry facility of  
8 IMRB (INSERM, U955).

9  
10 **Author contributions:** C.B. designed and performed neuronal transcriptomic study, immunohistochemistry,  
11 *in vitro* and *in vivo* experiments, and wrote the first draft of the manuscript, A.M. performed  
12 immunohistochemistry analysis, A.J. and P.G. analyzed microarray and transcriptomic data, D.G. performed  
13 data analysis including single cell RNAseq and manuscript revision, N.N. analyzed statistical data, F.S., P.M.,  
14 P.S. performed immunohistochemistry work and patient samples, V.F., F.V., D.D., M.A., S.T., A.D.T. and  
15 A.L.V. involved in writing the manuscript and discussions, Y.A. A.L.V. and V.F. supervised PAIR-prostate  
16 program and human histopathological data, J.D. and Y.H.K. supervised the entire project, designed  
17 experiments, wrote the manuscript and manuscript revision. All authors read and approved the final  
18 manuscript.

### 19 20 **Ethics approval and consent to participate**

21 Prostate tissue samples were collected as part of an Institutional Review Board approved protocol at Henri  
22 Mondor Hospital in France.

23 All mouse experiments were performed according to guidelines on animal care and with appropriate  
24 institutional certification of ethical comity and conducted in compliance with European Community.

### 25 **Consent for publication**

26 Not applicable

### 27 28 **Availability of data and materials**

29 Data generated and analyzed during this study are included in this published article and its supplementary  
30 information files. Other datasets used during the current study are available from the corresponding author on  
31 reasonable request.

32 HTA2.0 data have been deposited to the NCBI Gene Expression Omnibus (GSE200879).

33  
34 **Competing interests:** The authors declare that they have no competing interests

35 **Funding:** This research was supported by INSERM and the Ministère de l'Enseignement Supérieur et de la  
36 Recherche (MRT Scholarship) and by grants from Association pour la Recherche sur les Tumeurs de la  
37 Prostate (ARTP) and the Institut National du Cancer (INCa) through the PAIR-prostate program.

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## 1 FIGURE LEGENDS

2 **Figure 1. The NE profile is increased in CRPC compared to HNPC.** **A.** Heatmap showing differential  
3 regulation of 1849 genes across 13 CRPC and 54 HNPC patients (Fold-change  $\geq 1.5$ ; p-value  $\leq 0.05$ ). See  
4 also Supplementary Tables S2 and S3. **B.** Functional bar graph from DAVID gene ontology analysis  
5 (<https://david.ncifcrf.gov>) of Mondor dataset CRPC upregulated genes. P values are represented as bars. **C.**  
6 **and D.** Left panels show violin plots of abundance of mRNA transcripts in (C) AR signaling (**29**), or (D)  
7 NEPC signature (**2**). Dots represent patients; diamonds and solid lines represent mean and 95% confidence  
8 interval, respectively. See also Supplementary Tables S4 and S5. Right panels show immunohistology staining  
9 for (C) PSA or (D) SYP protein expression in HNPC and CRPC samples respectively. Scale bars, 100 $\mu$ m. **E.**  
10 Scatter plot shows significantly up-regulated genes associated with “Neurogenesis” pathway (GO:0022008)  
11 in Mondor dataset CRPC compared to HNPC samples. See also Supplementary Table S6. **F.** Box plot shows  
12 gene expression of *NRP1* in 54 HNPC and 13 CRPC tumors (Mondor Dataset), as measured by transcriptomic  
13 array. **G.** Representative IHC for NRP1, PSA and SYP in HNPC and different CRPC tumors. Scale bars,  
14 50 $\mu$ m. See also **Fig. S1**.

15 **Figure 2. NRP1 promotes NED through regulation of the AR axis.** **A.** Left. Photos of LNCaP cultures,  
16 control (Left), after androgen deprivation (Right). Scale bars, 200 $\mu$ m. Middle. Western blot analysis of NRP1,  
17 AR, PSA and NE markers CHGA and  $\beta$ -Tubulin III in LNCaP cells over time after androgen depletion. Right.  
18 qPCR of *NRP1*, *KLK3* and NE marker *NSE* in control (light blue) and androgen-deprived LNCaP (dark blue)  
19 at Day 4 after androgen deprivation. **B.** Western blot of NRP1, AR, PSA and NE marker  $\beta$ -Tubulin III from  
20 LNCaP cells after androgen-depletion (CS-FBS) followed by DHT treatment at indicated doses and times  
21 (96h). **C.** Western blot of NRP1, AR, PSA,  $\beta$ -Tubulin III from LNCaP-NE cells during DHT treatment over  
22 time (see indicated doses and times). **D.** Western blot of NRP1 and other proteins in LNCaP cells after siRNA  
23 knockdown of AR. Non-targeting siRNA is siCTL. **E.** Western blot of NRP1 and other proteins after treatment  
24 of LNCaP cells with stated concentrations of enzalutamide. **F.** NRP1 promoter activity in LNCaP cells after  
25 AR pathway inhibition (CS-FBS and enzalutamide) or activation with DHT as measured by luciferase assay.  
26 **G.** Western blot for NE markers and AR axis proteins from LNCaP cells stably overexpressing NRP1-  
27 containing vector compared with empty vector (LNCaP-vector). **H.** Western blot of NE markers in LNCaP-  
28 NE cells following siRNA knockdown of NRP1.

29 **Figure 3. NRP1 promotes NED through the PKC pathway.** **A.i.** Phosphorylation status of all screened  
30 proteins based on a phospho-specific protein microarray analysis. **A.ii.** Phospho-specific protein microarray  
31 data shows fold change of indicated phosphoproteins in NED after normalization to total protein expression.  
32 **B.** Western blot of LNCaP during NED (upon androgen depletion) shows phosphorylation of Pan-PKC (S660).  
33 **C.** Western blot shows Pan-PKC (S660) phosphorylation from LNCaP, C4-2 and 22Rv1 cells stably  
34 transfected with NRP1 vs empty vector. **D.** Scatter plots show correlation between *NRP1* and *PRKCD* mRNA  
35 from Mondor clinical cohort. **E.** Western blot of PKC isoforms from PC3 cells transfected with siNRP1 or

1 siCTL. Bar graph (Right) shows relative protein levels as % control. **F.** Western blots of anti-NRP1 or control  
2 IgG LNCaP-NE Immunoprecipitates blotted for PKC $\alpha$ , PKC $\delta$  or NRP1 (Left panels). Total lysate blotted for  
3 PKC $\alpha$ , PKC $\delta$  or NRP1 shown in the right panel. Data are represented as mean  $\pm$  SEM; p value by two-tailed  
4 unpaired t test. \*\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . **G.** Western blot of PKC isoforms and NE markers  
5 in LNCaP-NE cells transfected with non-targeting siRNA (siCTL) or PKC $\alpha$ , PKC $\delta$ , PKC $\epsilon$  siRNA. Bar graph  
6 (Right) shows relative protein levels of CHGA or SYP as % control.

7 **Figure 4. PKC promotes cell survival in NED.** **A.i.** Western blot for NE markers in LNCaP-NE after  
8 treatment with DMSO (Left), enzastaurin (Right) for 4 days at 5 $\mu$ M. **A.ii.** Bar graph shows relative protein  
9 levels of CHGA, SYP in DMSO- or Enzastaurin-treated LNCaP-NE as % of control. **B.** Line graph shows  
10 timeline of cell viability of stably transfected LNCaP cells overexpressing NRP1 (squares) or with empty  
11 vector (circles) after enzastaurin treatment. **C.** Bar graph shows viability of LNCaP-NE cells after transfection  
12 with siRNA targeting PKC $\alpha$ , PKC $\delta$  and PKC $\epsilon$  or non-targeting siRNA (siCTL). **D.** Line graph shows LNCaP  
13 tumor size over time in nude mice after LNCaP ectopic xenografting, castration and daily treatment with  
14 Enzastaurin (squares) or no treatment (circles). N=7 for all conditions. See Materials and Methods for details.  
15 Data are represented as mean  $\pm$  SEM; p value by two-tailed unpaired t test. \*\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P$   
16  $< 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

17 **Figure 5. NRP1 overexpression leads to docetaxel resistance that is reversed through PKC inhibition.**  
18 **A.** Left: Dot plot comparisons of *NRP1* mRNA expression in mCRPC samples (n=118) prior to (Left) or after  
19 (Right) Taxane treatment. Data taken from Stand Up To Cancer (SU2C)/Prostate Cancer Foundation (PCF)  
20 Dream Team dataset (43). Data analyzed using cBioPortal. Right: Western blot of NRP1 in LNCaP cells 72h  
21 after docetaxel treatment. **B.** Dose-response curves show viability of stably transfected LNCaP clones  
22 overexpressing NRP1 or control vector after 72h incubation with docetaxel (doses indicated on X axis). **C.**  
23 Bar graph shows % apoptotic LNCaP cells stably overexpressing NRP1 (dark blue) or control vector (light  
24 blue) after 72h incubation with different doses docetaxel (doses indicated on X axis). **D and E.** Dose-response  
25 curves of (D) LNCaP-NE or (E) PC3 cells after docetaxel treatment at indicated doses with (Squares) or  
26 without (Circles) 5 $\mu$ M enzastaurin. **F.** Time course of PC3 tumor volume increase from PC3 cells ectopically  
27 xenografted into nude mice treated daily with glucose 5% (Circles, n=6), enzastaurin (Squares, n=5), weekly  
28 with docetaxel (Blue Triangles, n=6) or a combination of treatments (enzastaurin and docetaxel, Green  
29 Triangles, n=8). See Materials and Methods for details.



# Figures

Figure 1.

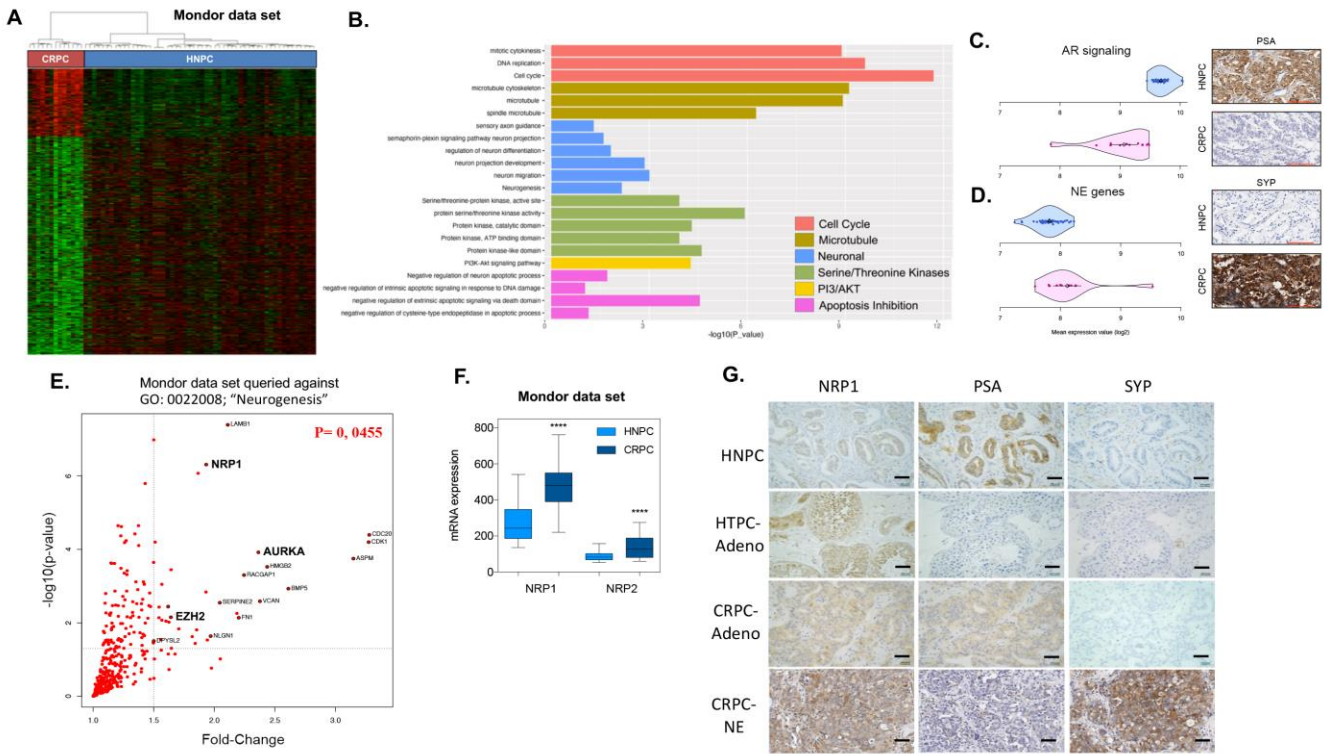


Figure 2.

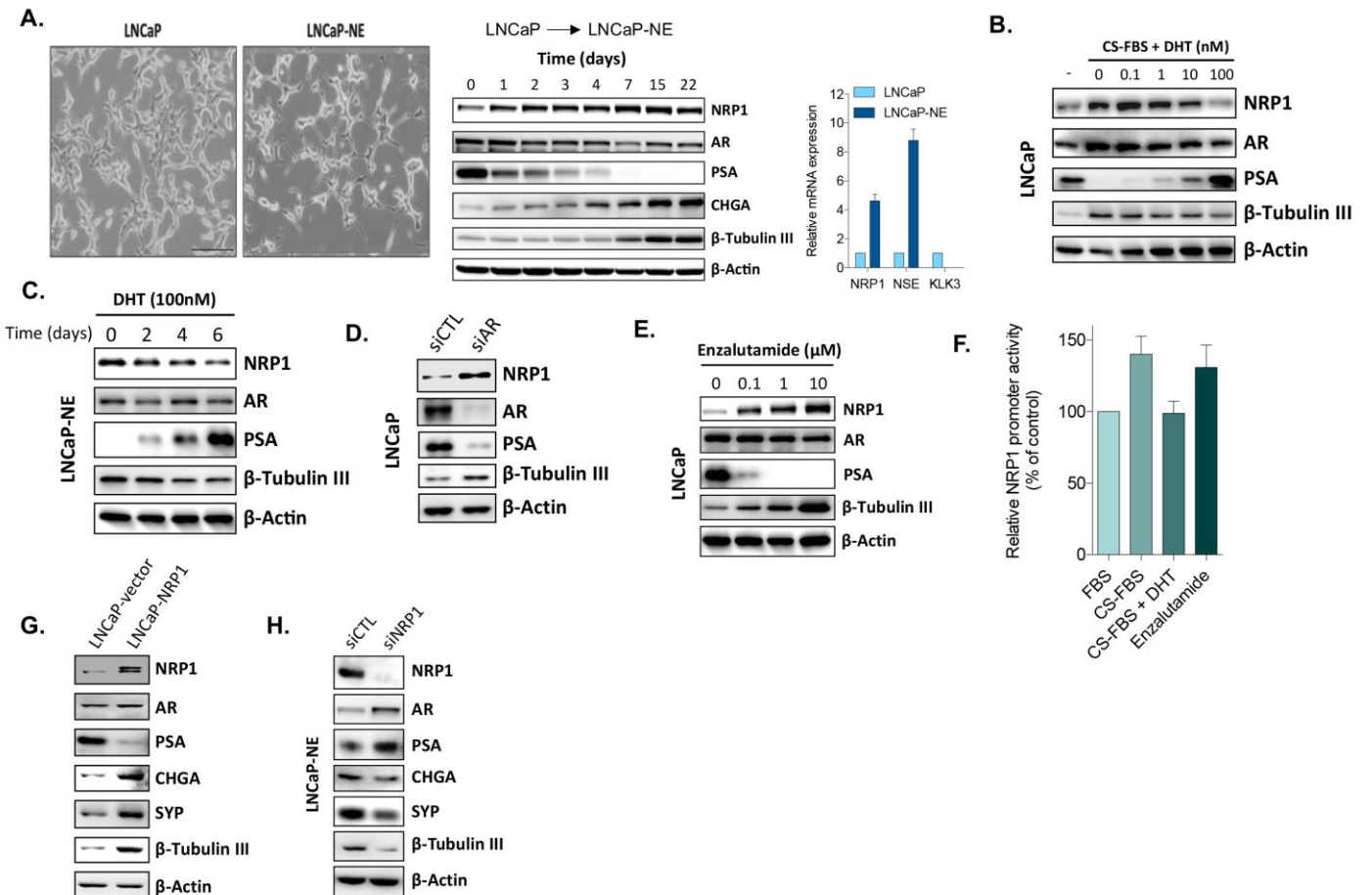


Figure 3.

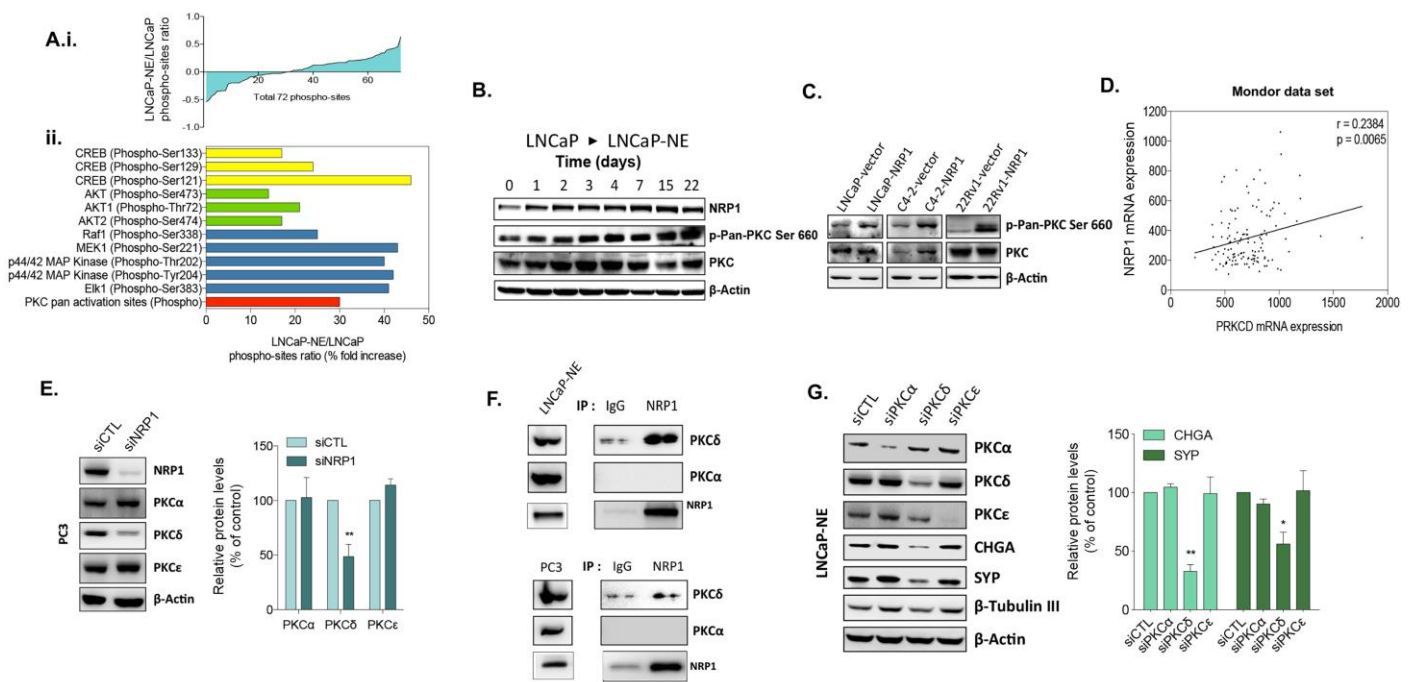


Figure 4.

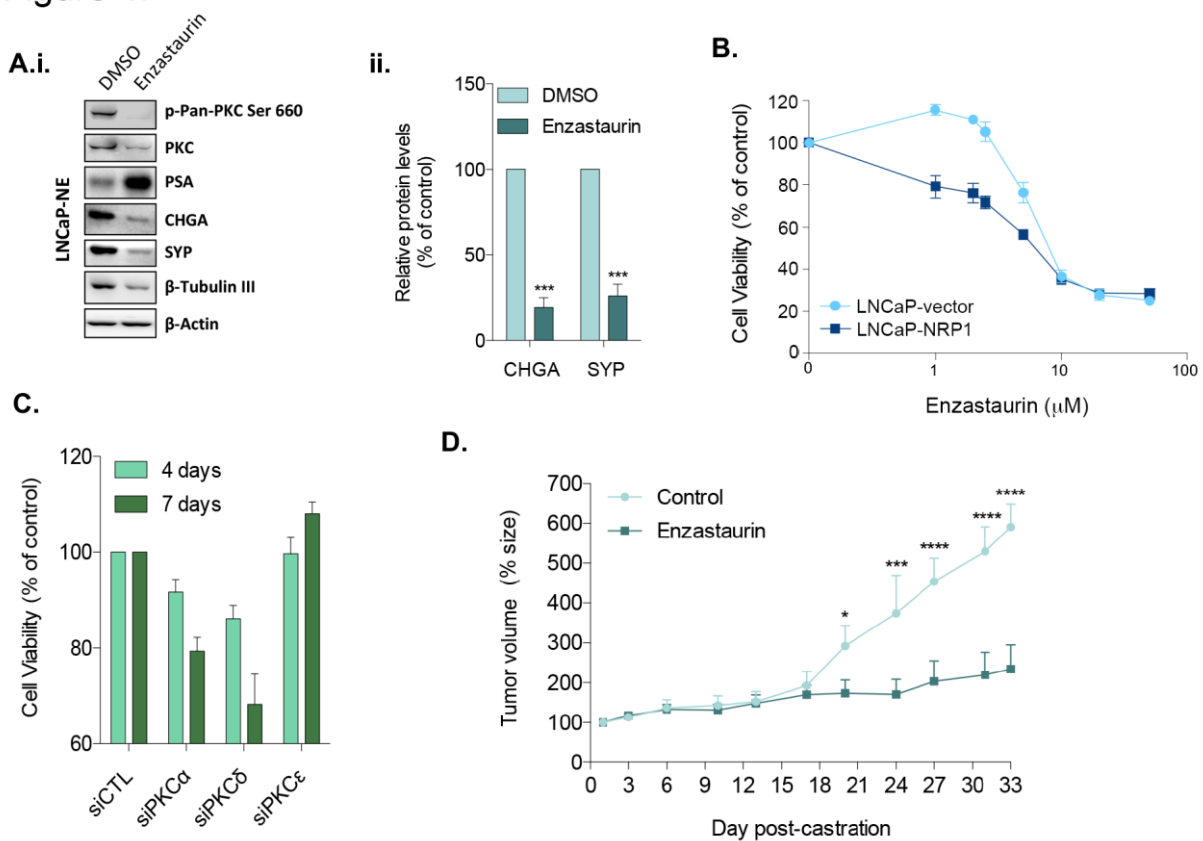
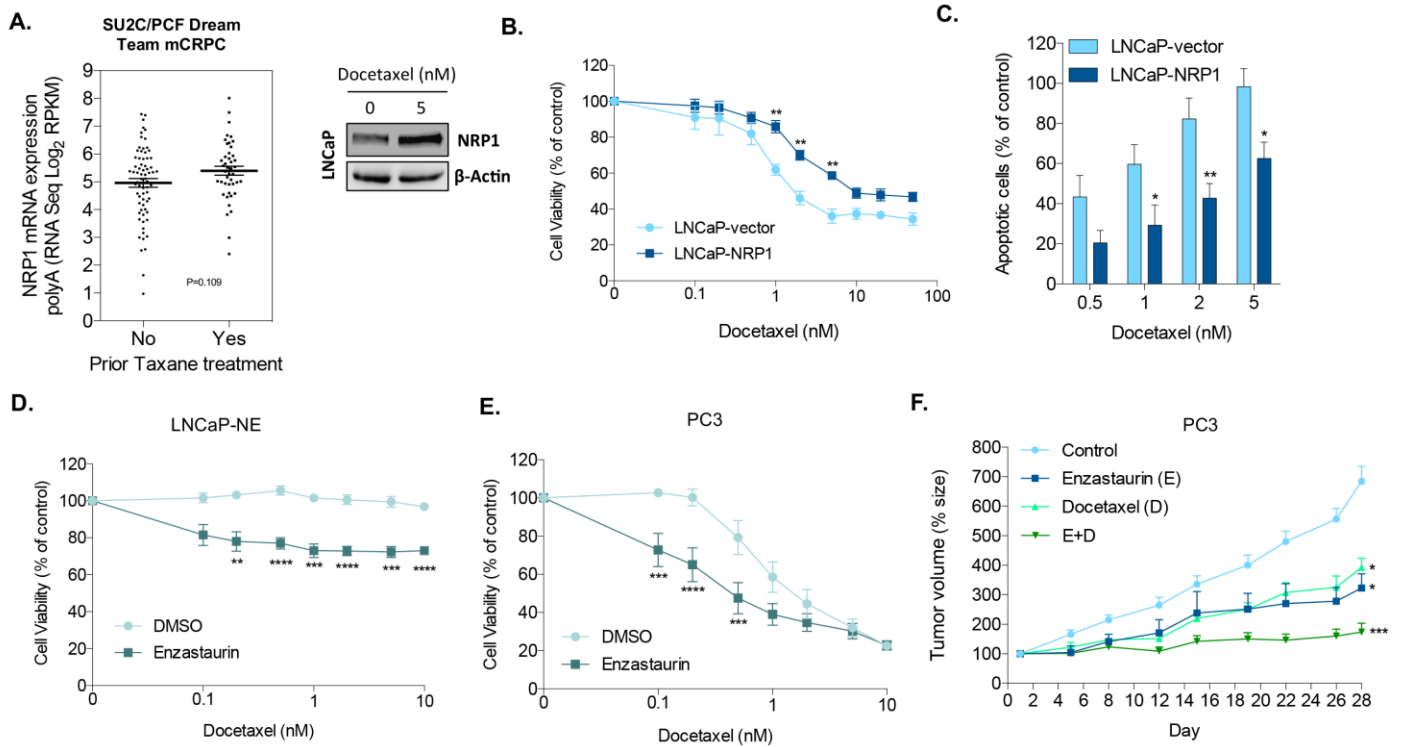
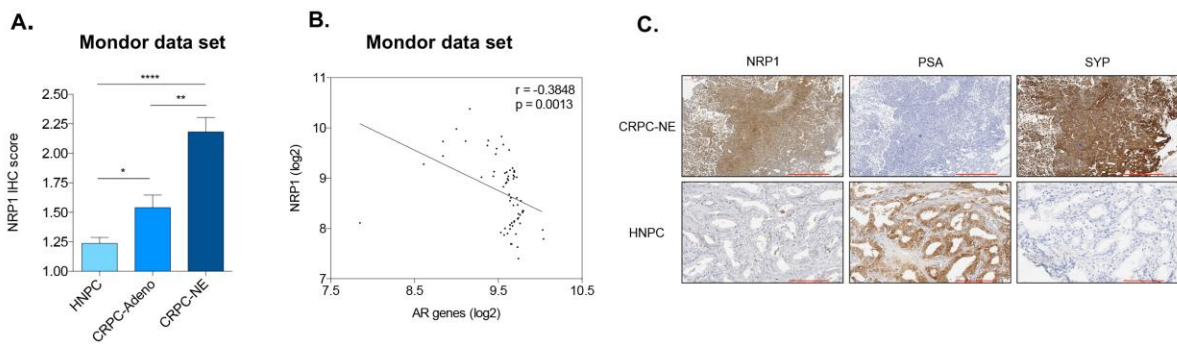


Figure 5.



## Supplementary Figures

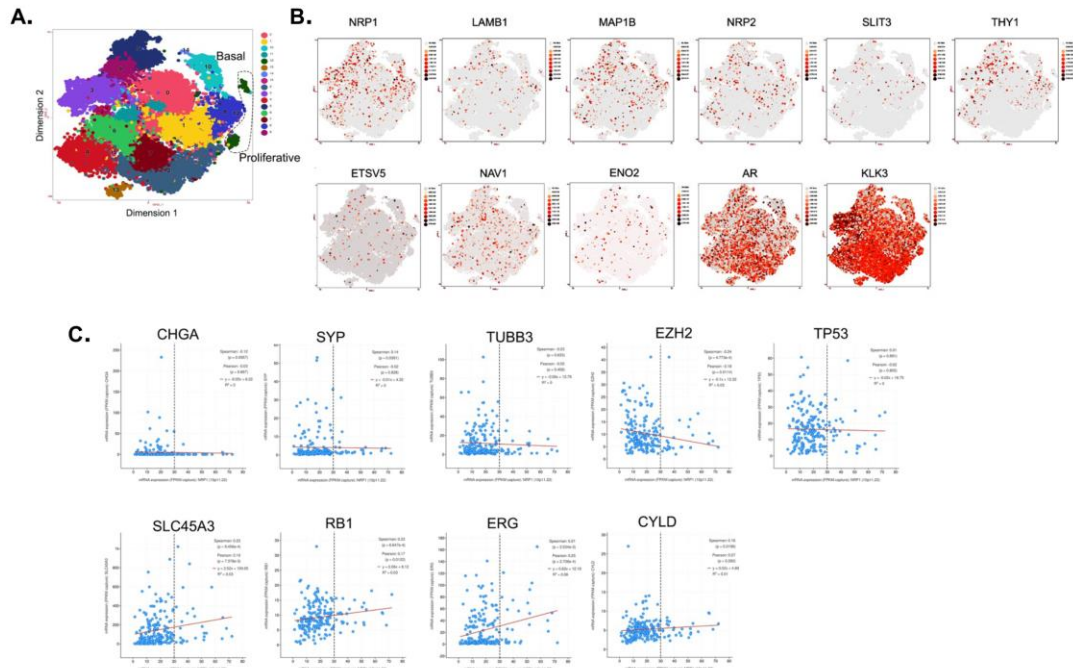
Fig. S1



**Supplementary Figure 1: NRP1 expression is associated with PCa progression, inversely related to AR signature and associated with a NED differentiation in human prostate carcinoma.**

**A.** IHC score for NRP1 protein expression in 169 HNPC, 27 CRPC-Adeno and 21 CRPC-NE. Statistical analyses used a two-tailed  $\alpha = 0.05$  level of significance, \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ . **B.** Scatter plot shows correlation between *NRP1* mRNA expression and AR signature from Mondor HNPC and CRPC cohort. **C.** Representative IHC for NRP1, PSA and SYP in a HNPC vs CRPC-NE tumor.

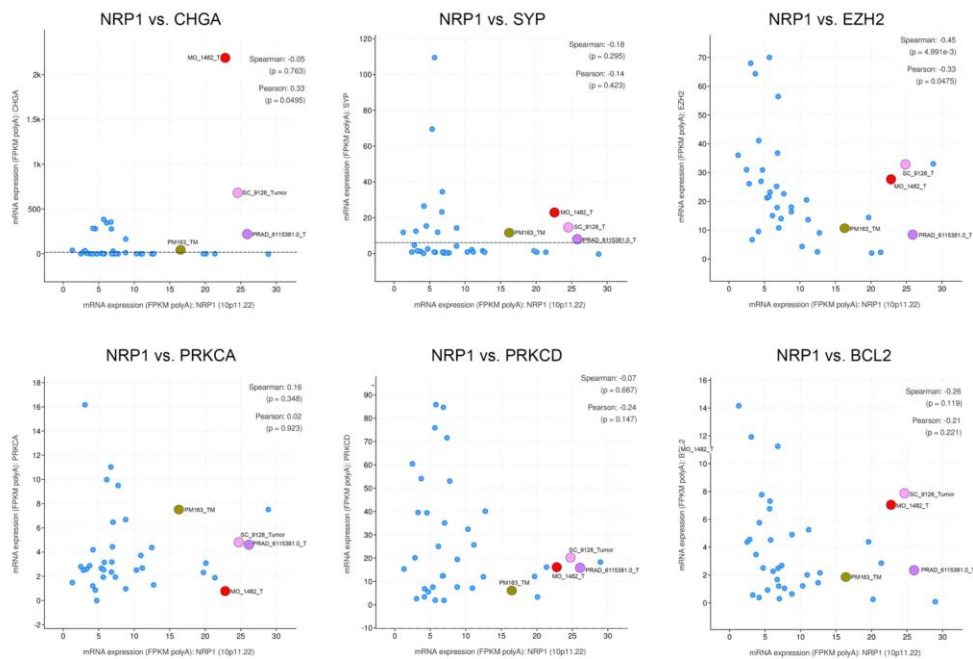
Fig. S2



**Supplementary Figure 2. scRNAseq analysis of 13 primary tumors confirms NE phenotype in most luminal clusters.**

**A.** TSNE plot shows clustering of malignant cells from integration analysis of 13 tumor biopsy samples across 12 patients (25). **B.** FeaturePlots show expression of selected genes across clusters. Data analyzed using Epithelial PradCellAtlas tool ([www.pradcellatlas.com](http://www.pradcellatlas.com)). **C.** Dotplots comparing co-expression of NRP1 (x axis) with defined genes (Y axis) in the SU2C-PCF dataset (208 samples, ref 32) using cBioportal tools (<https://www.cbioportal.org>).

Fig. S3

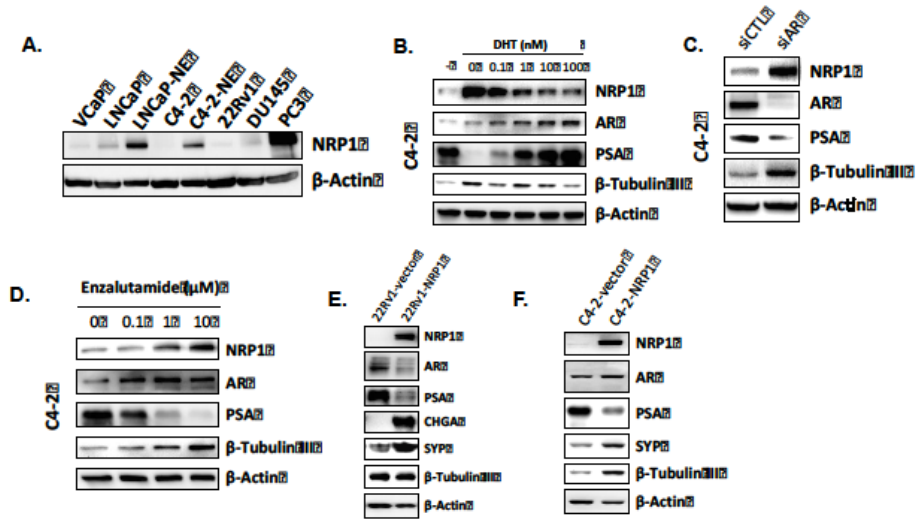


**Supplementary Figure 3. Analysis of a High NEPC Score cohort from the SU2C-PCF dataset reveals a subset of NRP1<sup>+</sup> cells.**

Dotplots compare co-expression of NRP1 (x axis) with defined genes (Y axis) from a subset of patients (32) with high NEPC scores from the SU2C-PCF dataset (39 samples, ref 32) using cBioportal tools (<https://www.cbioportal.org>). Dotted lines in “CHGA” and “SYP” plots mark cutoffs for positivity. Colored dots correspond to the adjacent sample name and are magnified in size to represent putative NRP1<sup>+</sup> candidates within the proposed true NE cohort. See Table S7 for clinical details.



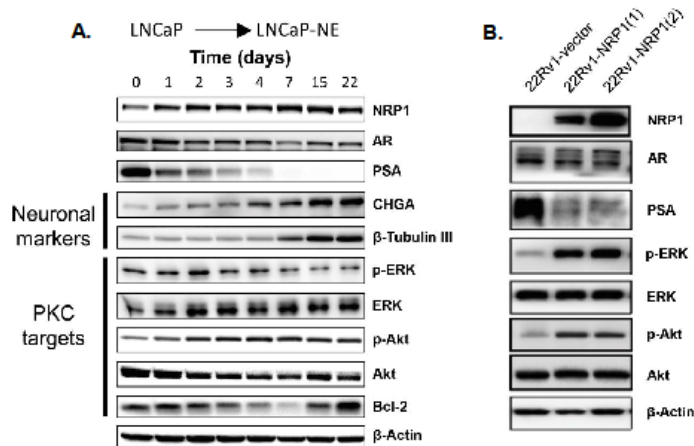
**Fig. S4**



**Supplementary Figure 4: NRP1 promotes NED through regulation of AR axis in a variety of PCa cell lines.**

A. Western blot showing NRP1 protein expression in two PCa androgen-dependent cell lines (VCaP, LNCaP), four androgen-independent cell lines (C4-2, 22Rv1, DU145, PC3), and two neuro-transdifferentiated cell lines upon androgen depletion (LNCaP-NE and C4-2-NE). B. Western blot of NRP1, AR, PSA,  $\beta$ -Tubulin III in C4-2 cells treated with DHT at indicated doses for 48h. C. Western blot shows NRP1 and other protein expression in C4-2 cells after treatment with AR siRNA or non-targeting siRNA. D. Western blot shows NRP1 and other protein expression after enzalutamide treatment in C4-2 cells. E and F. Western blots of NRP1, AR, PSA, NE markers CHGA, SYP,  $\beta$ -Tubulin III in stably transfected (E) C4-2 and (F) 22Rv1 cells overexpressing NRP1 (Right lanes). Cells transfected with empty vector (Left lanes).

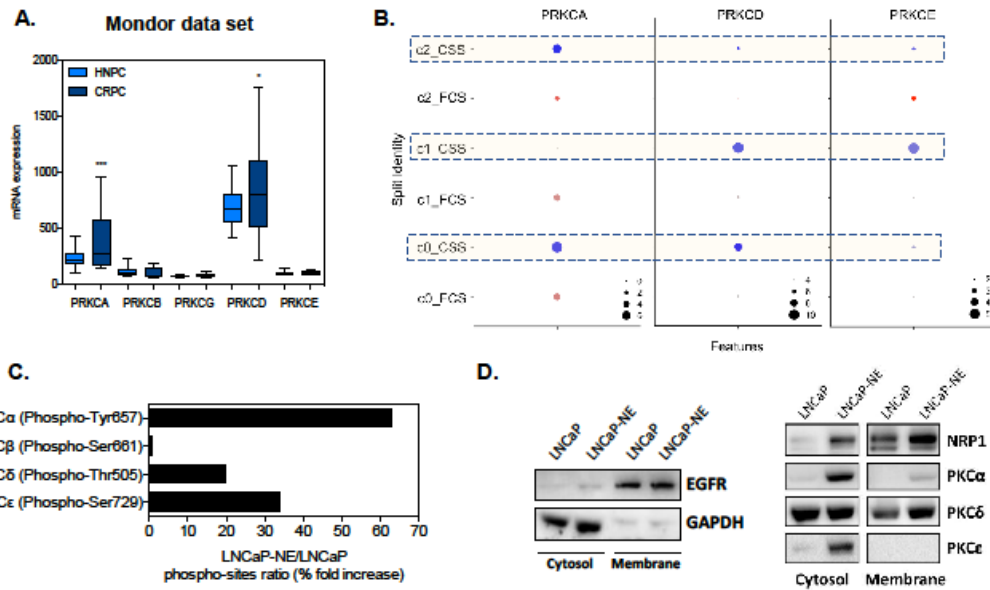
**Fig. S5**



**Supplementary Figure 5. Upregulation of PKC downstream targets in LNCaP-NE (A.) and after NRP1 over-expression (B.).**

A. Western blot of neuronal markers and PKC-downstream targets in LNCaP cells examined over 22 days following androgen depletion. B. Western blot of PKC-downstream targets in stably transfected 22Rv1 cells overexpressing 2 different NRP1 vectors (22Rv1-NRP1(1 or 2) or with empty vector (22Rv1-vector).

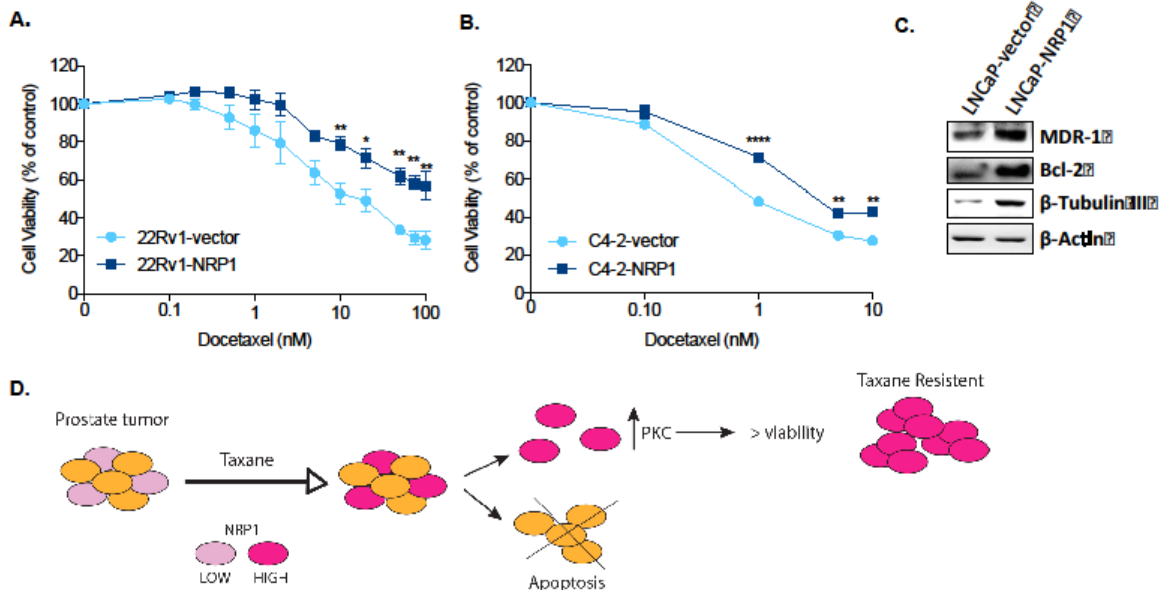
**Fig. S6**



**Supplementary Figure 6. Confirmation of transcription, phosphorylation and membrane localization of PKC isoforms in the NE phenotype.**  
**A.** Transcriptomic array results of mRNA expression levels of PKC isoforms in Mondor dataset. **B.** DotPlots show relative expression of defined genes in scRNAseq analysis from GSE205765 (See Materials and Methods for details). FCS (red) and CSS (blue) represent control and hormone-resistant groups, respectively. Scale represents percent expression. Boxed regions highlight CSS clusters. **C.** Bar graph shows antibody microarray levels of phospho-PKC pathway isoforms in LNCaP-NE compared to LNCaP. **D.** Western blots of NRP1, PKC $\alpha$ , PKC $\delta$ , PKC $\epsilon$  from subcellular cytosol (Left panels) or membrane (Right panels) fractions of LNCaP or LNCaP-NE cells.

1

**Fig. S7**



**Supplementary Figure 7. NRP1 overexpression confers docetaxel resistance to 22Rv1 and C4-2 cells in vitro.**  
**A and B.** Dose-response curves show cell viability in 22Rv1 (A) or C4-2 (B) clones stably overexpressing NRP1 (squares) or control vector (circles) after 72h incubation with docetaxel (indicated doses on X axis). **C.** Western blot shows MDR-1, Bcl-2 and  $\beta$ -Tubulin III expression in LNCaP cells stably transfected with NRP1 (LNCaP-NRP1) or empty vector (LNCaP-vector). **D.** Model. In this schematic, a subset of pre-treatment tumor cells undergoes increased NRP1 expression upon ADT. NRP1 upregulation drives PKC activation for downstream survival (and drug resistance) in NE tumors. It remains unknown if NRP1 is expressed by a subset or all pre-treatment cells. Further, it remains unknown whether the subset of NRP1<sup>+</sup> NE tumor cells observed in Fig S3 reflects a time point in drug resistance or one of several mechanisms used for drug resistance..

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