SUPPLEMENTAL METHODS

Cell lines and cell culture

RWPE-1 cells were maintained in Keratinocyte Serum Free Medium (K-SFM) (Invitrogen, Catalog Number 17005-042) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) under recommended conditions. LNCaP, C42B, PC3 cells were maintained in RPMI 1640 media (UCSF cell culture facility) and Du145 cells were cultured in MEM media, each supplemented with 10% fetal bovine serum (FBS) (Atlanta biologicals) and 1% penicillin/streptomycin (UCSF cell culture facility). Immortalized non-transformed prostate epithelial cell line (BPH1) (1) and NCI-H660 (CRl-5813) (2) was maintained in RPMI 1640 media and HITEs media, respectively, each supplemented with 5% FBS, and 1% penicillin/streptomycin.

Stable cell line generation

We generated stable clones overexpressing control/ *MYCN* by transfecting LNCaP/AR and C42B cell lines with *MYCN* construct/control vector (Genecopoeia) followed by puromycin selection. shRNA-mediated *TP53* and *RB* dual knockdown was performed in LNCaP/AR cells by co-transfecting with sh*TP53* pLKO.1 puro (Addgene, #19119) (3) and pSLIK sh human *RB* 1534 hyg (Addgene, #31500) lentiviral particles followed by selection in puromycin and hygromycin.

pLKO.1 puro (4) and shp53 pLKO.1 puro (3) were a gift from Bob Weinberg (Addgene plasmid # 8453) and pSLIK sh human Rb 1534 hyg was a gift from Julien Sage (Addgene plasmid # 31500). pLenti-BRN4-GIII-CMV (#LV268693) and pLenti-BRN2-GIII-CMV (#LV268681) were procured from Applied Biological Materials and used for overexpressing *BRN4* and *BRN2*, respectively in LNCaP-AR and C42B cell lines followed by puromycin selection. shRNA construct for *BRN2* knockdown was procured from Santa Cruz Biotechnologies (#29837-SH) and was used to transfect NCI-H660 cells and LNCaP-AR cells as per manufacturer's protocol.

ENZ treatment in LNCaP cells

We used following two methods to induce NED in LNCaP cells: (i) LNCaP cells were grown under androgen-depleted conditions by culturing in RPMI medium with 10% charcoal-dextran stripped FBS (C/D FBS) for 4 days. These conditions have been shown to induce robust NED, following which cells were treated with DMSO/ 20μM enzalutamide for 48 hours. As a control, LNCaP cells grown under regular conditions (RPMI medium+10% FBS) were included. (ii) Alternatively, LNCaP cells were treated with DMSO/1μM enzalutamide for 1 month in regular media.

Immunoprecipitation (IP)

Cells were lysed in buffer containing 250 mM NaCl, 50 mM HEPES pH 7.5, 0.1% Nonidet P40, 5 mM EDT and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The lysate was incubated with anti-BRN4 antibody (Sigma #31984) or normal rabbit IgG (Cell Signaling) and protein A/G-Sepharose beads (Santa Cruz Biotechnology #2002) overnight at 4°C. The protein A/G-Sepharose beads were washed with the lysis buffer. The proteins were eluted from the beads by boiling in Laemmli buffer (BioRad #161-0737) and were then separated by SDS-PAGE and analyzed by Western blot.

Clonogenicity assays

Cells were counted, seeded at low density (5000 cells/well of 6-well culture dish) and allowed to grow until visible colonies appeared. Cells were stained with crystal violet, and colonies were counted.

Migration and invasion assays

Control inserts (for migration) or Matrigel inserts (for invasion) (BD Biosciences) were used for performing *in vitro* transwell migration and invasion assays as per manufacturer's protocol. Briefly, cells were counted and 40,000 cells in a volume of 300 μl serum-free medium were placed on Matrigel inserts and control inserts, respectively. Cells were allowed to migrate for 24 hrs at 37°C. Following

this, cells were removed from the top of the inserts and cells that migrated/invaded though the polycarbonate/basement membrane were fixed, stained, counted under light microscope.

Immunoblotting analyses

EVs/cells were lysed with RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 0.1% SDS, and 1.0% NP-40] containing protease inhibitor cocktail (Roche). Protein lysates (10μg for EVs and 60μg for cells) were loaded onto a 4–20% Tris-glycine gradient gel (Biorad) and Western blotting was performed as per standard protocols. The following antibodies were used: BRN4 (Sigma, HPA31984), CD63 (System Biosciences, EXOAB-CD63A-1), CD9 (Cell Signaling Technology, 13174), TSG101 (System Biosciences, EXOAB-TSG101-1), GRP94 (Cell Signaling Technology, 2104), BRN2 (Cell Signaling Technology, 12137), AR (Cell Signaling Technology, 5153), SOX2 (Cell Signaling Technology, 3579), Vinculin (Sigma, SAB4503069), ENO2 (Invitrogen, PA5-27452), SYP (RM-9111-S, Thermo Fisher Scientific), CHGA (Invitrogen, MA5-13096), EZH2 (Abcam, ab3748), N-Myc (Santa Cruz Biotechnology, sc-56729), TP53 (Santa Cruz Biotechnology, sc-126), RB (Cell Signaling Technology, 93135) and GAPDH (Santa Cruz Biotechnology, sc-32233).

Quantitative real-time PCR

Mature mRNAs were assayed using the TaqMan Gene Expression Assays (Applied Biosystems) in accordance with the manufacturer's instructions. TaqMan assays used were *BRN1* (assay ID Hs00275987_s1), *BRN2* (assay ID Hs00271595_s1), *BRN4* (assay ID Hs00264887_s1), *NKX3.1* (Hs00171834_m1), *KLK3* (Hs02576345_m1), *CD44* (Hs00174139_m1), *AR* (Hs00171172_m1) and *GAPDH* (Hs99999905_m1). The comparative Ct method was used to calculate the relative changes in gene expression on the 7500 Fast Real Time PCR System.

Isolation of EVs from serum samples

Serum derived EVs were isolated from 250µL of serum using the Total exosome isolation reagent (Life Technologies, Cat. No. 4478360) as per manufacturer's instructions and as described in (5). Serum samples (0.5ml-1ml) were obtained from Prostate Cancer Biorepository Network (PCBN) and stored at -80°C till processed. No additives were used for serum isolation. Thawing the serum and all the further steps for EVs/exosomes isolation were carried out at 4°C. Briefly, serum samples were spun at 2000x g for 30 minutes followed by addition of 0.2 volumes of exosome isolation reagent to clarified supernatants. Samples were incubated at 2°C to 8°C for 30 min and exosomes/EVs were recovered by centrifugation at 10,000x g for 10 minutes at room temperature. Pellets were resuspended in 70-125µL of PBS and purified EVs were stored at -80°C till further processing for downstream application.

EV isolation from cells

Cells were cultured in RPMI 1640 media or MEM media each supplemented with 1% pen-strep and 10% of exosome depleted FBS (Gibco, #A27208-01) at 37°C, 5% CO_2, 95 % air for 48 hours before collecting the conditioned medium for EV/exosome isolation. Cells were grown in 10cm-dishes in 7 ml of media. At the time of collection, cells were ~50% confluent. All the further steps for EV/exosome isolation were carried out at 4ºC. Conditioned medium was centrifuged at 2000xg for 30mins (Fisher Scientific accuSpin 1R, fixed angle rotor) and the supernatant was transferred to 10K ultracel filter (Amicon ultra-15 #UFC901024) to concentrate the supernatant following which 0.5 volume of total exosome isolation reagent (#4478359) was added to it. After an overnight incubation at 2-8°C, the supernatant was centrifuged at 10,000xg for 1hour (Fisher Scientific accuSpin Micro 17R, fixed angle rotor). The pellet was re-suspended in 70-100µL of PBS and isolated EVs/exosomes were stored at -20ºC till further processing for downstream application.

EV and cellular RNA extraction from cultured cells

EV/exosomal RNA and cellular RNAs were prepared using a Exosomal RNA Purification kit (Norgen Biotek) and RNeasy kit (Qiagen) respectively as per manufacturer's instructions. The quantity and quality of the RNA was determined by a Agilent Bioanalyzer 2100 (Agilent Technologies) with a nano RNA chip as per the manufacturer's instructions.

GW4869 treatment

PC3 and LNCaP cells were cultured in media with exosome depleted FBS and treated with 10μM (or 20μM, if indicated) of DMSO/GW4869 for 48 hours. Conditioned media and cells were harvested for isolation of exosomal and cellular fractions, respectively.

EV quantitation and size determination

To confirm the integrity of EV preparations, particle sizes and concentrations were evaluated using Nanoparticle Tracking Analysis (NTA) on a NanoSight LM10 instrument (Malvern Instruments) as per manufacturer's instructions and as described in (5).

***In vitro* uptake assays**

For uptake assays, EVs were isolated from LNCaP cells treated with DMSO/1μM enzalutamide for 1 month. Purified and characterized EVs were labelled with SYTO RNASelect green fluorescent stain (Thermofisher Scientific) (6) as per manufacturer's protocol. Briefly, isolated EVs were incubated with 10μM stain for 20 minutes at 37°C, followed by removal of unincorporated dye from the labeled EVs with EV/exosome spin columns (Life Technologies). Labelled EVs (40μg/ml) were incubated with parental LNCaP cells. As a negative control, parental LNCaP cells were incubated with media with no EVs. After 48 hours, cells were harvested for RNA and protein or fixed for fluorescence microscopy.

5-Ethynyl Uridine (5EU) labelling of nascent RNAs

Control/BRN4 overexpressing LNCaP-AR cells were treated with 0.4mM 5EU (Life Technologies) for 24 hours in exosome- depleted RPMI media. Conditioned media was collected followed by isolation

of EVs containing EU-labelled mRNAs using exosome isolation reagent (#4478359). Following characterization of EVs by NTA analyses, EVs were added at a concentration of 40μg/ml in culture medium and incubated with recipient cells (parental LNCaP-AR) for 48 hours. Recipient cells were harvested and labelled 5EU mRNAs from recipient cells was purified by the Click-iT Nascent RNA Capture Kit (#C10365, ThermoFisher) following the manufacturer's recommended protocol. Briefly, RNA is used in a copper catalyzed click reaction with an azide modified biotin which creates a biotin-based handle for capturing nascent RNA transcripts on streptavidin magnetic beads. 5 micrograms of RNA were used for purification using this kit following which purified 5EU-labelled mRNA was reverse transcribed. Uptake of BRN4 was assessed by real time PCR analyses. GAPDH was used as a control.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 min and processed for immunofluorescent (IF) staining with BRN2 antibody (Cell Signaling, Cat no. 12137). For IF, cells were incubated overnight at 4°C with 1:200 dilution of BRN2 antibody, followed by detection using Alexa Fluor anti-rabbit IgG (594) secondary antibody (Invitrogen, Catalog no. A21207). Nuclei were counterstained with DAPI. All immunofluorescence pictures were taken at x40 magnification on a Nikon Eclipse Ti-S fluorescence microscope equipped with NIS-Elements-D software.

Engineering of EVs with low *BRN2* expression

For engineering EVs with modulated *BRN2* levels, we employed control shRNA (Cat no. sc-108060)/ *BRN2* shRNA (Cat no. sc-29837) vectors (Santa Cruz Biotechnology) to stably transfect NE cell line NCI-H660 (CRL-5813) (2). Following stable knockdown, EVs were collected from conditioned media of control shRNA vs *BRN2* shRNA transfected cells by culturing in media with exosome-depleted FBS for 48 hours.

Prostate Cancer Transcriptome Analyses

Correlation between BRN4 and BRN2/AR was determined in mCRPC cases in PCTA cohort (7) at www.thepcta.org.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1 Induction of neuroendocrine characteristics upon enzalutamide treatment and/or androgen withdrawal of LNCaP cell line

Control LNCaP cells were cultured in regular RPMI+10% FBS) or grown under androgen depleted conditions (RPMI medium with 10% C/D FBS) followed by treatment with either DMSO or 20 μM ENZ for 48 hrs.

A. Control/treated cells visualized by phase contrast microscopy.

B. Relative mRNA levels of various neuronal markers as assessed by real-time PCR. Neuroendocrine cell line, NCI-H660 was included as a positive control. Data were normalized to GAPDH control and represented as mean ± SEM.

C. Western blot analyses of neuronal markers after various treatments. GAPDH was used as a loading control.

Fig. S2 Correlation of EV-associated BRN4 and BRN2 with clinicopathological parameters of mCRPC

Correlation of EV-*BRN4* and EV-*BRN2* in CRPC-Adeno patients (cohorts 1+2) with clinicopathological parameters of the disease. P-values are based on Chi-square test.

Fig. S3 EV-associated BRN2 mediates neuroendocrine differentiation states in prostate cancer

A. Relative transcript levels of indicated genes in control (DMSO)/1μM ENZ EVs- treated parental LNCaP-AR cells as assessed by real-time PCR.

B. Knockdown of *BRN2* in NCI-H660 cells (left panel) and EVs (right panel) as assessed by real time PCR after transfecting with sh*BRN2* vs sh*CON* vectors.

C. Parental LNCaP cells were incubated with sh*CON* vs sh*BRN2* NCI-H660 EVs for 48 hours. After 48 hours, parental LNCaP cells were harvested for RNA followed by analyses of relative transcript levels of indicated genes by real-time PCR.

For A-C, real time PCR data were normalized to *GAPDH* control and represented as mean ± SEM.

Fig. S4 Release of oncogenic NE factors in prostate cancer EVs upon ENZ treatment

A. EVs were isolated from control (DMSO)/1μM ENZ treated LNCaP cells and subjected to Western blot analyses for indicated proteins.

B. LNCaP cells were cultured under regular conditions (control) or under androgen depleted conditions (C/D FBS) or treated with 10μM Enzalutamide in C/D FBS media. These treatments were followed by treatment with exosome inhibitor GW4869 for 48 hrs. EVs were extracted after various treatments followed by Western blot analyses for indicated proteins. CD9 was used as a positive control for EV lysates in A and B.

SUPPLEMENTAL TABLE LEGEND

Table S1 Clinicopathologic characteristics of metastatic CRPC patients (Cohort 1+2)

Table summarizing the age, race, Gleason score of primary tumor, final serum PSA, metastatic sites and prior therapies of clinical cohorts 1 and 2 used in the study.

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