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## Supplemental Information

# Androgen Receptor Is a Non-canonical Inhibitor of Wild-Type and Mutant Estrogen Receptors in Hormone Receptor-Positive Breast Cancers

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**Transparent Methods**

*Reagents*. TaqMan PCR primers and fluorescent probes, master mixes, Cells-to-Ct reagent, and lipofectamine were obtained from Life Technologies (Carlsbad, CA). Cell culture medium and charcoal-stripped fetal bovine serum (csFBS) were purchased from Fisher Scientific (Waltham, MA). FBS was purchased from Hyclone (San Angelo, TX). Vetspon dental cubes/sponges (Patterson Veterinary Supplies Inc., Cat. No. NC0654350) were obtained from Fisher Scientific (Waltham, MA). Details of vendors and catalog numbers are provided in the table below.

| Reagent | Vendor | Catalog number |
|---|---|---|
| Enzalutamide | Medkoo | 201821 |
| Enzalutamide | PharmaSys | TKn20120729 |
| AR antibody N20 Western blot | SantaCruz biotechnology | SC-816 |
| AR antibody PG21 Western blot/IHC | Millipore | 06-680 |
| GAPDH antibody Western blot | Sigma | G8795 |
| ER-α antibody | Cell Signaling | 8644 |
| DHT | Sigma | A8380-1G |
| Fulvestrant | Chemshuttle | 139028 |
| Fulvestrant | Tocris | 1047 |
| Taqman primers and probes | Life Technologies/Fisher | |
| pSer81 AR antibody | Millipore | 07-1375 |
| pSer118 ER antibody | Cell signaling | 2511 |
| PMA | R&D | 1201/1 |
| EGF | R&D | 236-EG-200 |
| Cell titer glo | Promega | G7572 |
| Estradiol | Tocris | 2824 |
| Estradiol pellet | Innovative Research of America | E-121 |
| AR antibody ChIP-Seq | Springbiosciences | E2724 |
| FOXA1 antibody ChIP-Seq | Abcam | 5089 and 23738 mixture |
| ER antibody ChIP-Seq | NEOMARKERS/Santa Cruz | MS-315-PABX (ER ab10 (TE111.5D11) and sc543 mixture |
| Lipofectamine | Life Technologies | 18324012 |
| Dual luciferase assay reagent | Promega | E1910 |
| Ku80 antibody for IHC | Cell Signaling | 2180 |

*Cell culture.* ZR-75-1 and COS7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in accordance with the ATCC recommendations. Cell lines were authenticated by short terminal DNA repeat assay (Genetica cell line testing laboratory).

*Growth assay.* Cells were plated in growth medium in 96 well plates. Cells were treated for the duration indicated in the figures. Medium was changed every third day. Cell viability was measured by counting the number of cells using Coulter counter.

*Tumor xenograft experiments.* All animal protocols were approved by The University of Tennessee Health Science Center (UTHSC) Institutional Animal Care and Use Research Committee (IACUC). Xenograft experiments were performed as previously published (Narayanan et al., 2014)**.** Wherever ovariectomy was described, the animals were ovariectomized in the institution under anesthesia and in accordance with the IACUC approved protocol. HCI-7, HCI-9, and HCI-13 (invasive lobular breast cancer) PDXs were kindly provided by Dr. Alana Welm (Huntsman Cancer Institute, Salt Lake City, Utah). HCI PDX tumor fragments (1 $mm^3$) were surgically implanted under the mammary fat pad in female NOD SCID Gamma (NSG) mice. HCI-7 PDX was performed in animals that were ovariectomized and supplemented with estradiol pellet. HCI-9 and HCI-13 PDX was performed in intact animals that were not ovariectomized. Tumor volume was measured once or twice weekly for HCI PDXs depending on their growth properties. At the end of the study, animals were sacrificed and tumors were excised, weighed, and stored for various analyses. HCI-7 PDX were performed twice, while HCI-13 PDX was performed five times. All the experiments reproduced the representative results shown in this manuscript.

*Patient specimen collection.* Specimens from breast cancer patients were collected with patient consent under a protocol approved by the UTHSC Institutional Review Board (IRB). The protocol number for the IRB approval is 14-03113XP. Specimens were collected immediately after surgery in RPMI medium containing penicillin:streptomycin and Fungizone and transported to the laboratory on ice. The tissues were finely minced and treated with collagenase for 2 hours. The digested tissues were washed with serum-free medium and frozen in liquid nitrogen in freezing medium (5% DMSO+95% FBS) or implanted under the mammary fat pad in female NSG mice.

*Sponge culture.* Patient specimens frozen in liquid nitrogen in freezing medium were used for sponge culture. Sponge cultures were performed in accordance with the protocol published earlier (Dean et al., 2012; Hu et al., 2016; Ochnik et al., 2014)**.** Tumors were sliced into small pieces (~1 $mm^3$) and incubated on pre-soaked gelatin sponges (5 fragments/sponge) in 12 well plates containing 1.5 mL medium (MEM+10% FBS+2 mM L-glutamine+10 µg/mL insulin+10 µg/mL hydrocortisone + penicillin: streptomycin). The cultures were performed in triplicates. Pooled samples (n=5 fragments/sponge) from each sponge constituted one sample. Medium was replaced the next day and treated as indicated in the figures. Tissues were harvested after 3 days of treatment, RNA extracted, and expression of various genes measured. Characteristics of the patient specimens used in PDX and in sponge cultures are provided in **Table ST1**.

*Microarray.* RNA from tumors was extracted and verified qualitatively and quantitatively. Total RNA (200 ng/sample; n=4/group) from each sample was amplified and labeled using the WT Plus

Kit from Affymetrix and processed according to Affymetrix protocol. The arrays (Human ST2.0, Affymetrix, Santa Clara, CA) were washed and stained on Affymetrix Fluidics station 450 and scanned on an Affymetrix GCS 3000 scanner.

Data from microarrays were normalized using Affymetrix Expression Console. Mean, Standard Deviation, and Variance were calculated across the groups. Fold Change from vehicle-treated samples was calculated, and a fold change of 1.5 was used as cutoff. Student's t-test was used to determine the significance and a cutoff of p value $< 0.05$ was used for significance discovery. False discovery rate was calculated using Benjamini & Hochberg method, and a cutoff for FDR $< 0.05$ was used to create a significant differential expression list. The gene candidate list was loaded to Ingenuity Pathway Analysis. Microarray experiments were performed at the UTHSC Molecular Resources Center (MRC), and data analysis was performed by the UTHSC Molecular Bioinformatics core facility.

*Chromatin immunoprecipitation assay (ChIP) -Sequencing (ChIP-Seq).* HCI-13 xenograft specimens were snap frozen and stored for ChIP-sequencing analysis. ChIP-Seq study was performed in vehicle or enobosarm-treated HCI-13 PDX grown in NSG mice. ChIP was performed with ER, AR, or FOXA1 (n=3-5/group) antibodies and genome-wide sequencing were performed on a NextSeq 500 sequencer. For ChIP, the protocol was based on existing procedures (Carroll et al., 2005) with some modifications. Briefly, the frozen xenograft tumors were sectioned and bifunctional cross-linking was performed at room temperature with 2 mM dissuccinyl glutarate (DSG) for 45 minutes followed by 10 minutes fixation with 1 % methanol-free formaldehyde. A standard SDS-based protocol was used, whole lysates were made from the tissues and were

sonicated using a Covaris E220 machine (Covaris Inc., Woburn, MA), for 20 minutes per sample (settings: 10% duty factor, 175 peak intensity power at 200 cycles per burst). ER, AR, or FOXA1 was immunoprecipitated, washed, and the complex eluted. The DNA-protein complex was reverse cross-linked by treating with proteinase K (1 µg/µl) and incubating at 65°C for 6 hours to overnight. After reverse cross-linking, precipitated and input DNA was purified using Minielute PCR purification columns (Qiagen).

For library preparation, Accel-NGS 2S Plus Library Kit (Swift Biosciences, Ann Arbor, MI) was used. For each library 2-10 ng DNA was used. After amplification, fragments of 200-600 bp were selected and cleaned using AmPure XP beads (Beckman Coulter, Indianapolis, IN) and analyzed on a Fragment Analyzer (Advanced Analytical, Ames, IA). For sequencing, NextSeq 500 sequencing platform (Illumina, San Diego, CA) was used. Human genome build 19 (hg19) was used as the reference genome. Sequencing data from ChIP experiments were aligned to the human genome using Bowtie (Langmead et al., 2009). For peak calling MACS2 was used (Feng et al., 2012). Significance is defined as regions that are greater than or less than 2 fold different in enobosarm-treated samples compared to vehicle-treated samples and had a $q<0.05$ for ER, and $q<0.05$ for AR and FOXA1.

*Data and software availability*: Accession number for the sequencing data from public deposition is GSE128018. Microarray data was deposited in GEO and the accession number is GSE126318.

*Statistics:* Statistical analysis was performed using GraphPad prism software (La Jolla, CA). Experiments containing two groups were analyzed by simple t-test, while those containing more than two groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey

post-hoc test. Microarray, phospho-proteomics, and ChIP-Seq statistical analyses are described under the respective methods.

All *in vitro* experiments were performed at least in triplicate with each treatment having an n=3. Data are represented as mean ± S.E. Statistics are represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

*Serum drug concentration measurement*. Blood was collected 24-30 hours after the last dose and serum was separated. One hundred microliters of serum was mixed with 200 µl of Acetonitrile/Internal Standard and added to the plates. A serial dilutions of the respective drug standards were prepared in 100 µl of rat serum with concentrations ranging 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, .97 and 0 nM. Standards were extracted with 200 µl of Acetonitrile/Internal Standard and added to 96 well plates. The analysis of the drugs was performed using LC-MS/MS system consisting of Shimadzu Nexera X2 HPLC with an AB/Sciex Triple Quad 4500 Q-Trap™ mass spectrometer. The separation was achieved using a $C_{18}$ analytical column (Alltima™, 2.1 X 100 mm, 3 µm) protected by a $C_{18}$ guard column (Phenomenex™ 4.6mm ID cartridge with holder). Various parameters are provided in the table below.

| | **Enobosarm** | **DHT** | **Enzalutamide** | **Fulvestrant** |
|---|---|---|---|---|
| **Run time (min)** | 2.5 | 4.0 | 6.0 | 2.5 |
| **Injection volume (µl)** | 10 | 10 | 10 | 10 |
| **Mobile phase** | | | | |
| **Channel A** | 95% acetonitrile + 5% water + 0.1% formic acid | 100% methanol + 0.1% formic acid | 95% acetonitrile + 5% water + 0.1% formic acid | 100% acetonitrile + 0.1% formic acid |
| **Channel C** | 95% water + 5% acetonitrile + 0.1 % formic acid | 100% water + 0.2% formic acid | 95% water + 0.1% formic acid + 5% water | 100% water |
| **A:B (%)** | 5:95 | 70:30 | 30:70 | 20:80 |
| **Mode** | Negative | Positive | Positive | Negative |

| | | | | |
|---|---|---|---|---|
| **Declustering Potential (DP)** | -190 | 31 | 101 | -150 |
| **Collision Energy (CE)** | -34 | 25 | 41 | -38 |
| **Cell Exit Potential (CXP)** | -9 | 10 | 12 | -19 |
| **m/z** | 362.29/184.6 | 291/255.2 | 465/209.1 | 605.2/427 |

*Transfection*. Transient transactivation studies were conducted in COS7 cells. Briefly, COS7 cells were plated in DME+5%csFBS w/o phenol red medium in 24 well plates. Cells were transfected with 0.25 μg ERE-E1b-LUC or pS2-LUC, pCR3.1 human ER-α (all three plasmids were gifts from Dr. Carolyn Smith, Baylor College of Medicine, Houston, TX), and CMV-renilla-LUC using lipofectamine reagent (Life Technologies). Cells were treated 24 hours after transfection and luciferase assay was performed 48 hours after transfection. Firefly luciferase values were normalized to renilla luciferase numbers.

ER-LBD competitive binding assay. ER-LBD competitive binding assay was performed as previously described [1].

*Immunohistochemistry*: Fourteen cases of invasive breast cancer, luminal B subtype, were chosen randomly from the formalin fixed paraffin embedded samples available from the tissue bank of the pathology department of Tohoku University Hospital. The luminal B classification of these samples was on the basis of having ERα expression greater than 1% and a Ki-67 labelling index of greater than 20 percent. The samples had variable levels of PR expression (Labelling Index, Average 48.9, Range 0-100) and other clinicopathological characteristics (Ki67, Average 38%, Range 20-48%; Nottingham Grade, I n=1, II n=10, III n=3). The use of these samples was

approved by the Tohoku University School of Graduate Medicine Ethic review board (2014-1-107). Blocks of tissue were retrieved and sectioned at a thickness of 3 μM and mounted on glass slides. In order to asses co-localization mirror image sectioning was used. The slides were then stained for ERα and AR (ERα, 1:50 dilution, Clone 6F11, Leica ; AR , 1:50 dilution, Clone AR441, Dako) using immunohistochemistry as previously described [2,3]. In order to determine the proportion of stroma and epithelia in xenografts, H&E staining of FFPE sections was undertaken. The slides were then analyzed both by a trained and experienced pathological researcher and digitally using Image J who was blind as to the ID of the sections. In brief, initially the slides were read by an experienced pathology researcher, the percentage stroma estimated and a representative photomicrograph taken. The color threshold function of Image J was used manually to define stromal areas on this photomicrograph and area as a percentage of total cellular area was calculated using the histogram function.

*Gene expression.* RNA extraction and cDNA preparations were performed using cells-to-ct kit. Gene expression studies were performed using TaqMan probes on ABI 7900 realtime PCR machine. For gene expression studies in cells, cells were plated in 96 well plates in charcoal-stripped serum containing medium. Cells were maintained in this medium for two days and then treated as indicated in the figures. RNA was extracted and cDNA synthesized using cells-to-ct-kit. Gene expression studies in tumor xenografts were performed by extracting the RNA using RNA-isolation kit from Qiagen. cDNA was synthesized and the expression of genes was quantified by realtime PCR using TaqMan primers and probes.

*Western blotting*. Tumors were added to appropriate volume of lysis buffer containing protease and phosphatase inhibitors and were completely disintegrated using bead-based fragmentation method using FastPrep FP120 (Thermo). Protein was extracted by three freeze-thaw cycles. Equal amounts of protein were fragmented on a SDS-PAGE and transferred to a nitrocellulose membrane. Western blot for various proteins was performed by standard method.

*Detailed immunohistochemistry protocol for ki67, AR, and ER*.

**Day 1:**

**Note**: The slides were placed in a rack and the following steps were performed. The paraffin was melted with heat until total melted using hair dryer.

**A) Deparaffinize and rehydrate:**

1. Xylene: 2 x 10 minutes
2. Xylene: 2 x 5 minutes
3. 100% ethanol: 1 minutes
4. 95% ethanol: 1 minutes
5. 70 % ethanol: 1minutes
6. 50 % ethanol: 1 minutes
7. Running cold tap water to rinse.

**B) Antigen retrieval**

Prepare: 1 ml of citrate Buffer (Citrate buffer 1M pH 6.0) + 100 ml distilled water

8. The slides were placed in a rack with citrate solution and autoclaved for 5 minutes at 121°C.
9. The rack was taken out and cooled down for 20 minutes in crushed ice.
10. Washed three times with PBS.

**C) Blocking buffer & Primary antibody**

11. The slides were drained and 2-3 drops of blocking buffer was applied (Goat, Histofine SAB-PO kit; Nichirei, Tokyo, Japan) and incubated at room temperature for 30 minutes in a humid chamber.

12. The slides were drained and 55 µl of primary antibody (mouse, monoclonal antibody, diluted 1/50 in BSA 0.5- $NaN_3$ 0.05%-PBS for AR and ERα, and diluted at 1/100 for KI67, Rabbit polyclonal antibody) was added and incubated overnight at 4°C in humid chamber.

**Note**: **AR** monoclonal mouse antibody, clone AR441, DAKO.
**ERα** monoclonal mouse antibody, clone 6F11, Leica [NCL-L-ER-6F11].
**KI67** monoclonal mouse antibody, clone MIB-1, DAKO M7240

**Day 2:**

**D) Block endogenous peroxidases**

13. The following day, the slides were washed three times in PBS.
14. Slides were soaked in a new rack filled with 0.3% $H_2O_2$ solution (50 ml methanol + 0.5 ml $H_2O_2$) for 30 minutes at room temperature.

**E) Secondary antibody**

15. Washed three times with PBS.
16. The slides were dried and 2-3 drops of secondary antibody (same specie as primary antibody) was added, and incubated in humid chamber for 30 minutes at room temperature.

**F) Revelation with DAB**

17. Rinsed with PBS three times.
18. The slides were dried and 2-3 drops of Streptavidin-Peroxidase was added and incubated 30 minutes at room temperature.
19. The slides were washed with PBS three times and plunged them 3-5 minutes in DAB solution for the revelation (10ml Tris-buffer 0.25M pH 7.0+ DAB 13mg solution complete to 50ml distilled water + 20 µl $H_2O_2$).

**G) Counterstaining**

20. Rinsed with tap water and counterstained in hematoxylin 4 minutes.
21. Rinsed with running tap water.

**H) Dehydration**

22. Rinsed with running tap water
23. Immediately dehydrated slides in 95% ethanol (2x), 100% ethanol (2x), cleared in 4 baths of xylene.

**I) Mounting the slides:**

24. The slides were mounted using an automatic cover slipper (Tissue Tek Glas, Sakura)

## Hematoxylin Eosin staining

1. The wax was melted using hair dryer.
2. The wax was removed in successive baths of xylene 2 x 10 minutes, 2 x 5 minutes.
3. Hydrated in 4 baths of ethanol 1 minute each (100%, 95%, 70%, 50%).
4. Rinsed with tap water in a bucket.
5. The slides were immersed in Hematoxylin Carazzi for 30 minutes.
6. Washed with running tap water.
7. Immersed quickly 3 times in 1% HCl-alcohol bath.
8. Rinsed in distilled water for 5 minutes.
9. Stained with 1% eosin for 15 seconds.
10. Washed in tap water then proceeded to four baths in ethanol, then four in xylene.
11. The slides were mounted using automatic cover slipper machine (Tissue Tek Glass, SAKURA).

## IHC-PARAFFIN PROTOCOL

**Ku80**

### Day 1:

**Note 1** : The slides were placed in a rack and the paraffin was melted with heat until total melted using hair dryer.

**Note 2**: Positive control: Human colon cancer.

**B) Deparaffinize and rehydrate:**

8. Xylene: 2 x 10 minutes
9. Xylene: 2 x 5 minutes
10. 100% ethanol: 1 minutes
11. 95% ethanol: 1 minutes
12. 70 % ethanol: 1minutes
13. 50 % ethanol: 1 minutes

14. Running cold tap water to rinse.

**B) Antigen retrieval**

Prepare: 1 ml of citrate Buffer (Citrate buffer 1M pH 6.0) + 100 ml distilled water

8. The slides were placed in a rack with citrate solution and autoclave for 5 minutes at 121°C.
9. The rack was taken out and cooled down for 20 minutes in crushed ice.
10. Washed three times with PBS.

**C) Blocking buffer & Primary antibody**

11. The slides were drained and 2-3 drops of blocking buffer (Goat, Histofine SAB-PO kit; Nichirei, Tokyo, Japan) was applied, incubated at room temperature for 30 minutes in a humid chamber.
12. The slides were drained and 55 µl of primary antibody (Rabbit, monoclonal antibody, diluted 1/150 in BSA 0.5- $NaN_3$ 0.05%-PBS) was added. Incubated overnight at 4°C in humid chambers

**Note**: **Ku80** monoclonal Rabbit antibody, clone C48E7, Cell signaling Technology #2180

**Day 2:**

**D) Block endogenous peroxidases**

13. The following day, the slides were washed three times in PBS.
14. The slides were soaked in a new rack filled with 0.3% $H_2O_2$ solution (50 ml methanol + 0.5 ml $H_2O_2$) for 30 minutes at room temperature.

**E) Secondary antibody**

15. Washed three times with PBS.
16. The slides were dried and 2-3 drops of secondary antibody (same specie as primary antibody Rabbit) were added, and incubated in humid chamber for 30 minutes at room temperature.

**F) Revelation with DAB**

17. Rinsed with PBS three times.
18. The slides were dried and 2-3 drops of Streptavidin-Peroxidase was added and incubated 30 minutes at room temperature.

19. The slides were washed with PBS three times and plunged them 4 minutes in DAB solution for the revelation (10ml Tris-buffer 0.25M pH 7.0+ DAB 13mg solution complete to 50ml distilled water + 20 µl $H_2O_2$).

**G) Counterstaining**

20. Rinsed with tap water and counterstain in hematoxylin 3 minutes.
21. Rinsed with running tap water.

**H) Dehydration**

22. Rinsed with running tap water
23. Immediately the slides were dehydrated in 95% ethanol (2x), 100% ethanol (2x), and clear in 4 baths of xylene.

**I) Mounting the slides:**

24. The slides were mounted using an automatic cover slipper (Tissue Tek Glas, Sakura)

**Reagents:**

**A) Phosphate Buffer Saline PBS 0.01M**

| | |
|---|---|
| $NaH_2PO_4$. $2H_2O$ | 4.5g |
| $Na_2HPO_4$. 12H2O | 32.27g |
| NaCl | 80g |

**B) Bovine Serum Albumin BSA**

| | |
|---|---|
| BSA | 1g |
| 10% $NaN_3$ | 1ml |
| 0.01M PBS | 200ml |

**C) 1 M Citrate Buffer pH 6.0**

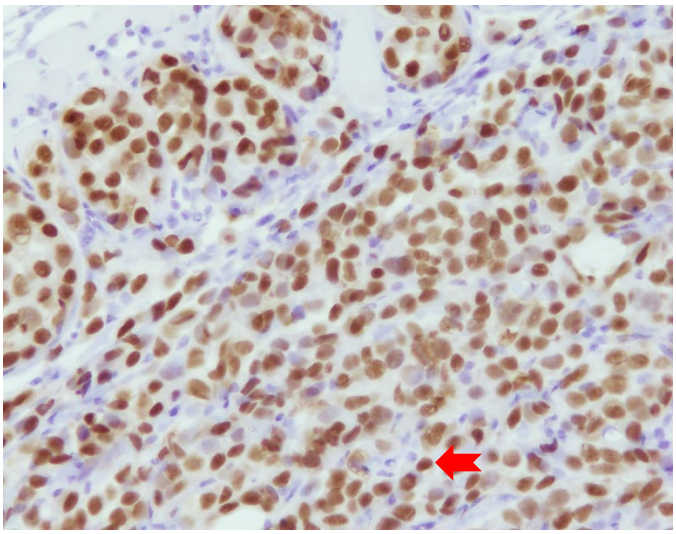
| | |
|---|---|
| A: Citric Acid monohydrate | 21g – distilled water 100ml |
| B: Trisodium citrate dehydrate | 147g – distilled water 500ml |
| Mix A 90ml + B 410 ml | |

**C) DAB**

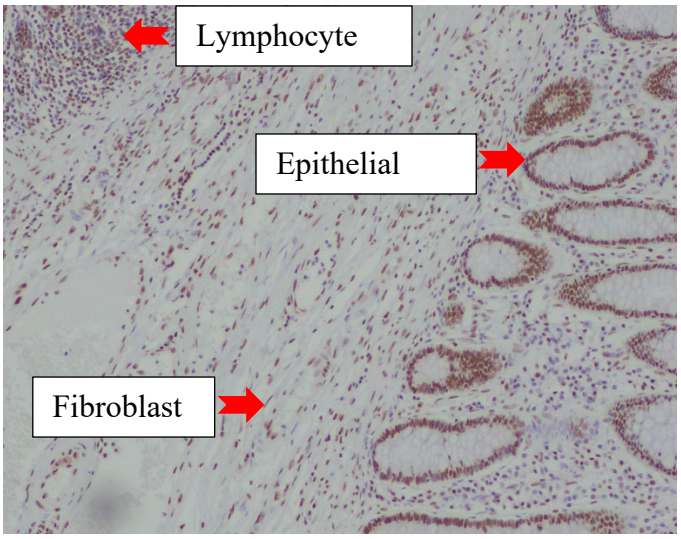
| | |
|---|---|
| 0.05 M Tris buffer (0.25M tris buffer 40 ml + 160 ml distilled water) | 200ml |
| DAB | 1 g |

Notes:

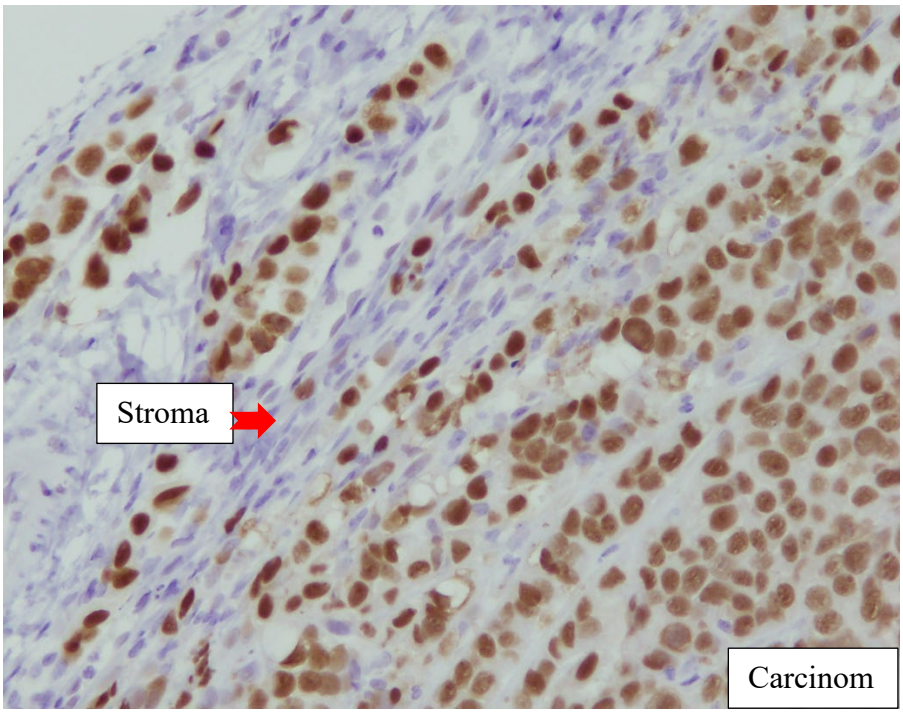
- The monoclonal antibody Ku80 detects the endogenous levels of total protein, nuclei localized.

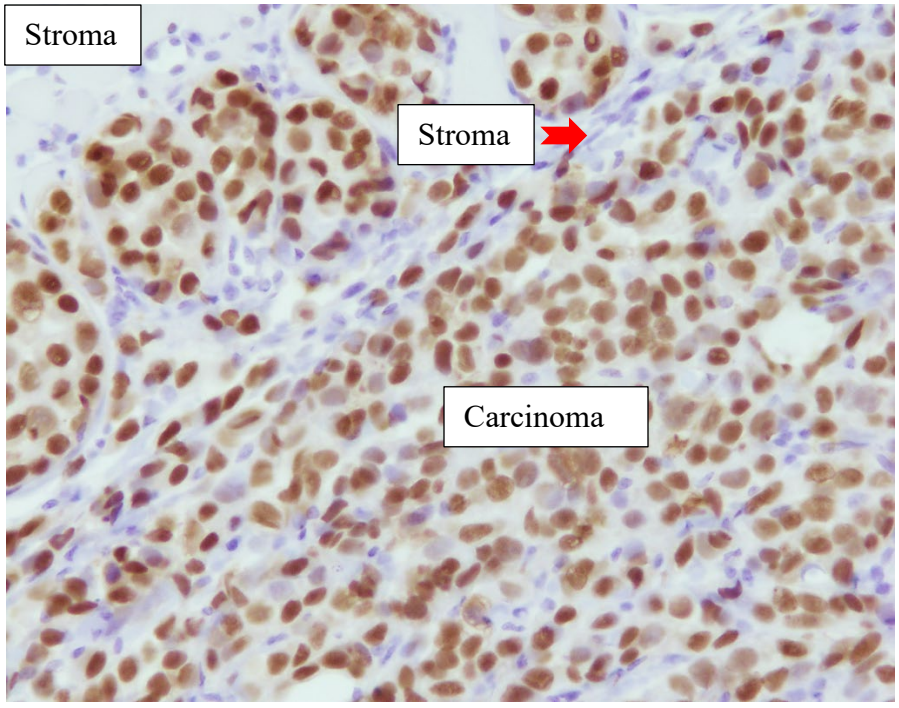


- In the human colon cancer positive control, the antibody stains positively the nuclei of epithelial cells, carcinoma and stroma cells (fibroblast, lymphocytes)



- In the PDx vehicle samples, the antibody stains positively the nuclei of human carcinoma cells, and not the stroma.



Reverse-phase protein array (RPPA)-based protein pathway activation mapping. Frozen samples from HCI-13 PDX treated with vehicle or enobosarm were cut into 8 µm cryosections and mounted on uncharged glass slides. Whole tissue lysates were directly prepared from the tissue sections using a 1:1 mixture of T-PER (Tissue Protein Extraction Reagent; Pierce, Rockford, IL) and 2X Tris-Glycine SDS Sample Buffer (Invitrogen, Carlsbad, CA) supplemented with 5% 2-mercaptoethanol [4]. Samples were boiled for 8 minutes and stored at -80°C until arrayed.

Samples and standard curves for internal quality assurance were printed onto nitrocellulose-coated slides (Grace Bio-labs, Bend, OR) using an Aushon 2470 arrayer (Aushon BioSystems, Billerica, MA). Selected arrays were used to estimate the amount of protein in each sample using a Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) protocol following manufacturer's instructions [4]. Remaining arrays were tested with a single primary antibody using an automated system (Dako Cytomation, Carpinteria, CA) as previously described [5]. Arrays were first incubated with Reblot Antibody stripping solution (Chemicon, Temecula, CA), followed by two washes in PBS, and I-block solution (Tropix, Bedford, MA) for 4 hours. Arrays were probed with a total of 174 antibodies targeting a wide range of protein kinases and their activation via phosphorylation (**Table ST2**). Antibodies specificity was tested using standard immunoblotting on a panel of cell lysates [4,6]**.** Selected arrays were stained with an anti-rabbit or anti-mouse biotinylated secondary antibody alone (Vector Laboratories Inc., Burlingame, CA and Dako Cytomation, Carpinteria, CA, respectively) and used as negative controls for non-specific binding/background subtraction.

The commercially available Signal Amplification System (CSA; Dako Cytomation) and a streptavidin-conjugated IRDye 680 secondary antibody (LI-COR Biosciences, Lincoln, NE) were used as signal detection methods. Images were acquired on the laser-based PowerScanner (TECAN, Mönnedorf, Switzerland), and data were analyzed using the MicroVigene software

Version 5.1 (Vigene Tech, Carlisle, MA) as previously described [5]. Intra and inter-assay reproducibility have been previously reported [7,8].

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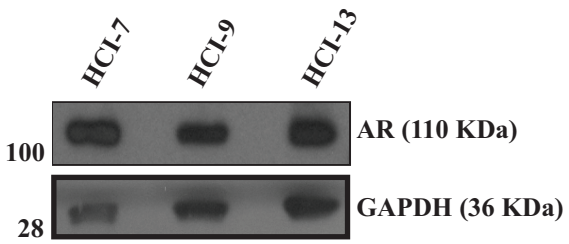
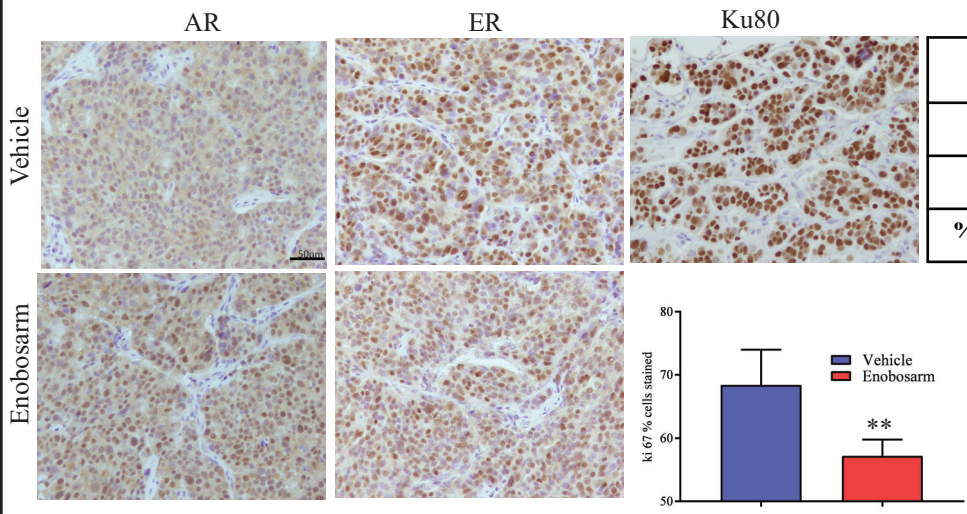
**Figure S1**




| | HCI-7 (n=5/group) Vehicle | |
|---|---|---|
| | Avg. | S.E. |
| % stroma | 8.5 | 1.83 |
| % epithelium | 91.5 | 1.83 |

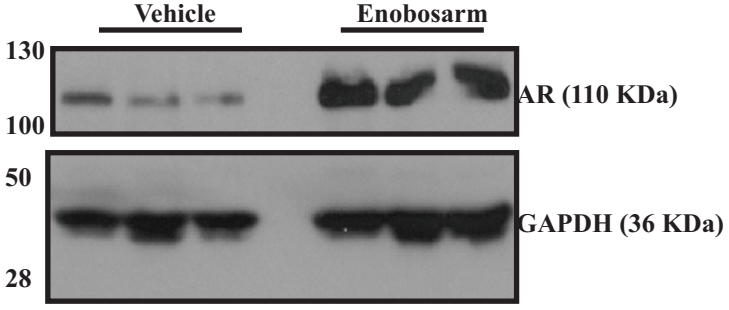


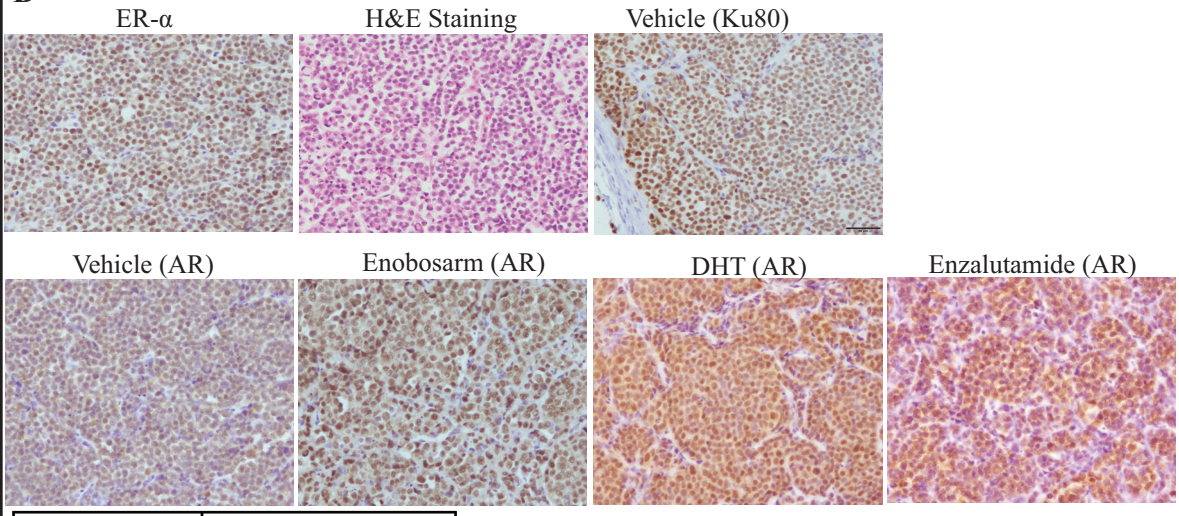

**Figure S1: HCI-7 PDX characteristics (related to Figure 1).** AR expression shown by Western blot in HCI-7, HCI-9, and HCI-13 PDXs. HCI-7 tumors from animals treated with vehicle or enobosarm (**Figure 1C**) were formalin-fixed and immunostained with the indicated antibodies. Representative images of n=4-5/group/stain are shown. Scale is provided in a representative image (50 μm). Ki-67 staining was quantified and represented as bar graph (n=5/group). AR and GAPDH Western blots in HCI-7 tumors from animals treated with vehicle or enobosarm is provided on the right. * $p<0.05$. AR-androgen receptor; ER-estrogen receptor; SARM-selective androgen receptor modulator; SRB-sulforhodamine B; mpk-milligram per kilogram body weight. Values are expressed as average ± S.E. from n=3-4/data point.

# Figure S2

**A**

| Drug | Concentration (nM) (n=4/group) | |
|---|---|---|
| | Avg. | S.E. |
| **Enobosarm** | 102102 | 3554 |
| **Enzalutamide** | 15398 | 1374 |
| **DHT** | 180 | 57 |
| **Fulvestrant** | 6399 | 1209 |

**B**




| | HCI-13 (n=6/group) Vehicle | |
|---|---|---|
| | Avg. | S.E. |
| **% stroma** | 16.3 | 2.97 |
| **% epithelium** | 83.7 | 2.97 |

**C**

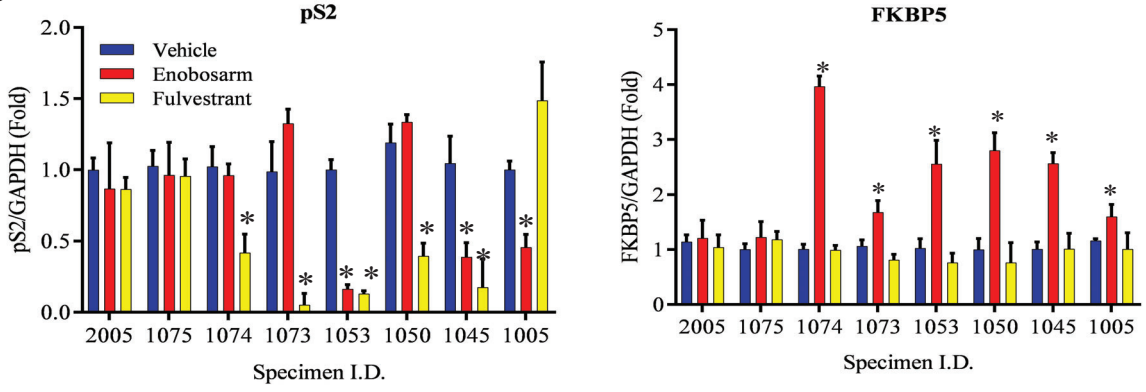


**Figure S2**: **HCI-13 PDX characteristics (related to Figure 1). A**. Drug concentration in the serum of HCI-13 tumor-bearing animals that were treated with enobosarm, enzalutamide, DHT, or fulvestrant using LC-MS/MS (n=4/group). **B**. HCI-13 tumors from animals treated with various drugs were formalin-fixed and immunostained with the indicated antibodies. Representative images of n=5/group/stain are shown. Scale is provided in a representative image (50 μm). Percent stromal and epithelial cells are shown in the table below. **C**. *Effect of enobosarm on ER-positive breast cancer patient specimens*. Breast cancer specimens obtained from patients were cultured on gelatin sponges (n=3; each n was obtained from 5 tumor fragments) in full serum containing growth medium. Tissues were treated with vehicle, 1 μM enobosarm, or 100 nM fulvestrant for three days. RNA was extracted from the tissues and expression of genes was measured by real time PCR and normalized to GAPDH.

**Figure S3**

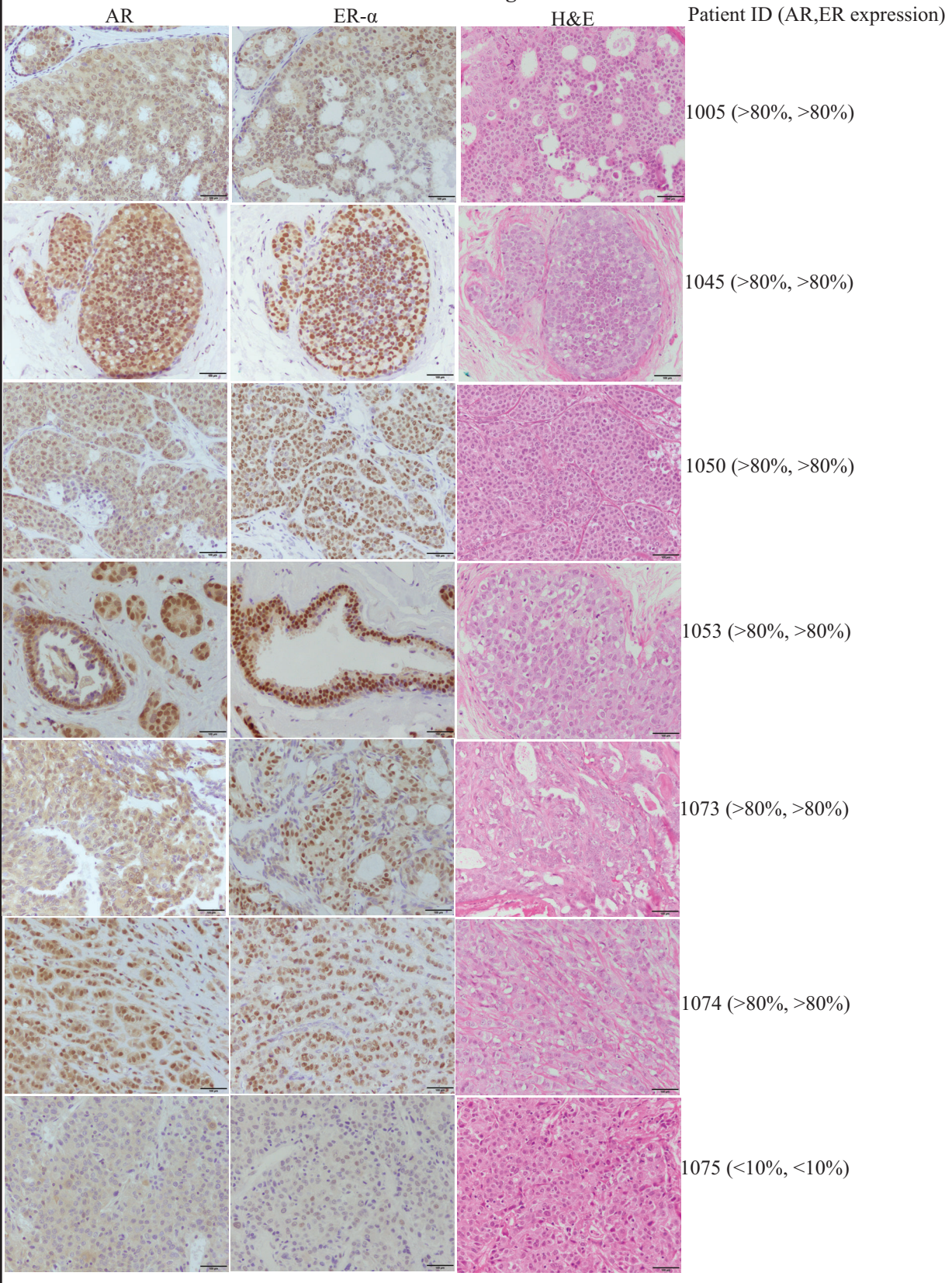
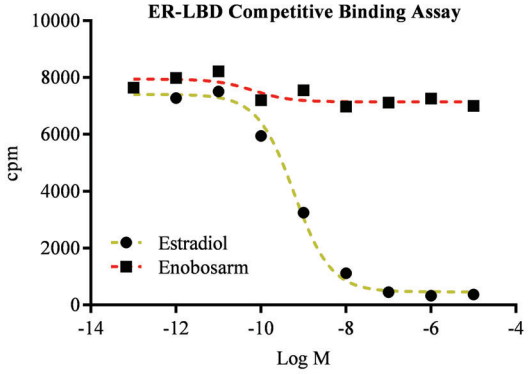



**Figure S3 (Related to Figure 1)**. Immunohistochemistry staining of patient specimens for AR, ER, and H&E staining. The numbers to the right of each stain indicates the patient ID and the percent cells stained for AR and ER. Scale is provided in a representative image (100 μm).

## Figure S4

A




B

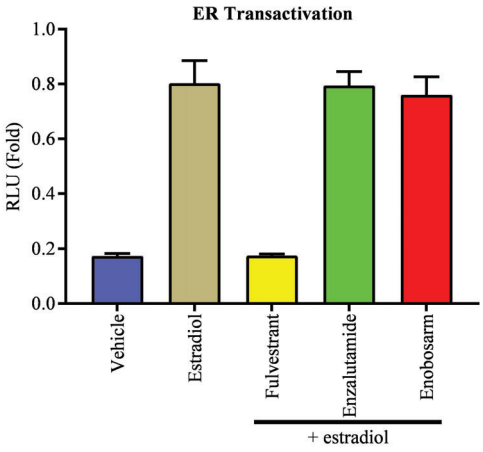



**Figure S4 (Related to Figure 3)**. **A**. ER-LBD competitive binding assay. ER-LBD protein made from bacterial expression plasmid was incubated overnight at 4°C with 1 nM $^{3}$H estradiol and increasing concentrations of cold estradiol or enobosarm. Amount of radioactive estradiol incorporated in the ER LBD (measure of the amount displaced by a cold ligand) is determined using scintillation counter. **B**. AR ligands have no effect on ER transactivation. 25 ng pCR3.1 hER-α, 0.25 µg pS2-LUC, and 10 ng CMV renilla-LUC were transfected into COS7 cells using lipofectamine. Cells were treated 24 hours after transfection with 0.1 nM estradiol alone or in combination with 100 nM fulvestrant, 10 µM enobosarm, or 10 µM enzalutamide. Luciferase assay was performed 48 hours after transfection. Firefly luciferase values were normalized to renilla luciferase values (n=3/treatment).

# Figure S5

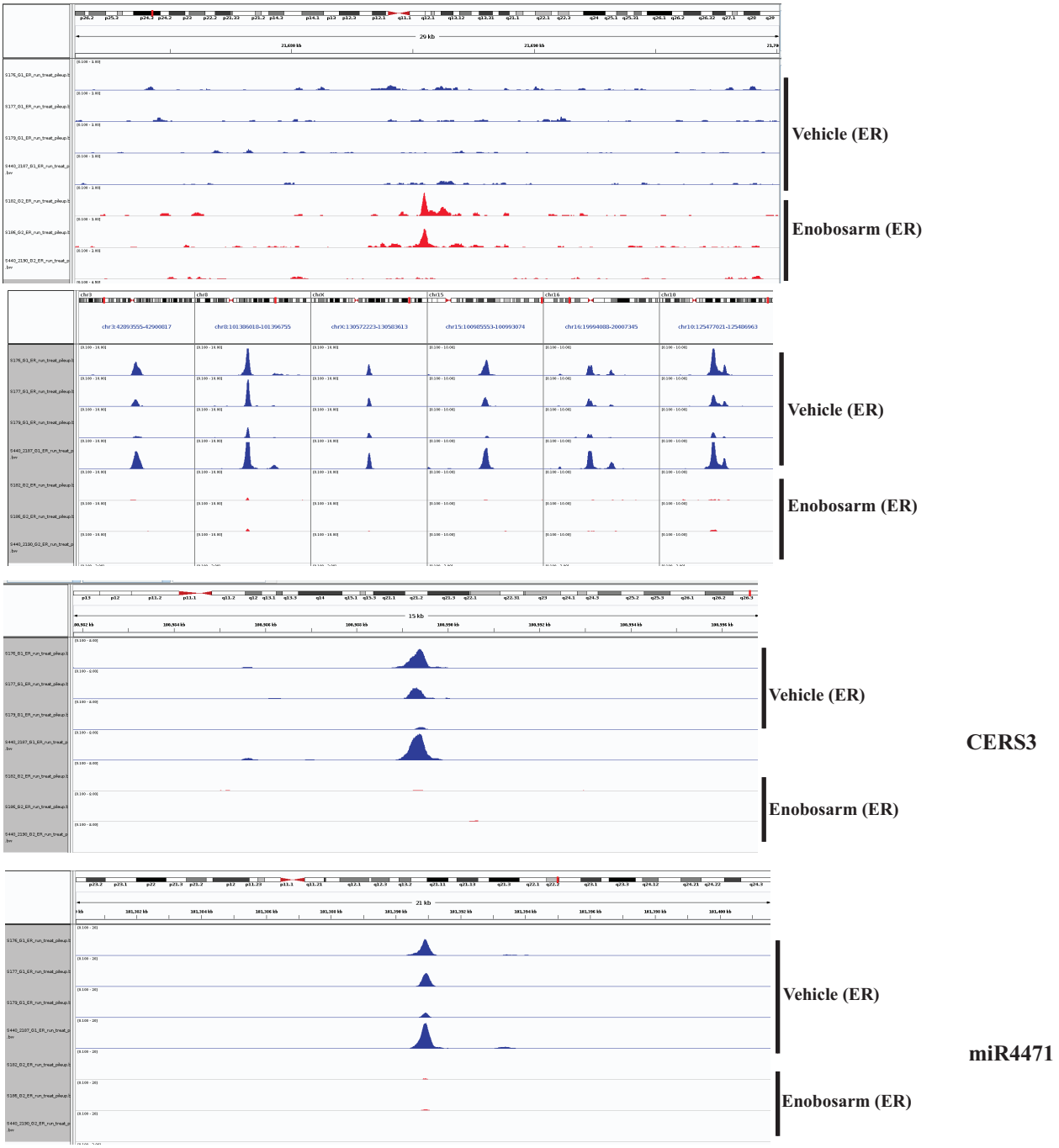



**Figure S5 (Related to Figure 3): Representative ER ChIP-Seq peaks in the regulated regions of genes**. Venn diagram of overlapping sites.

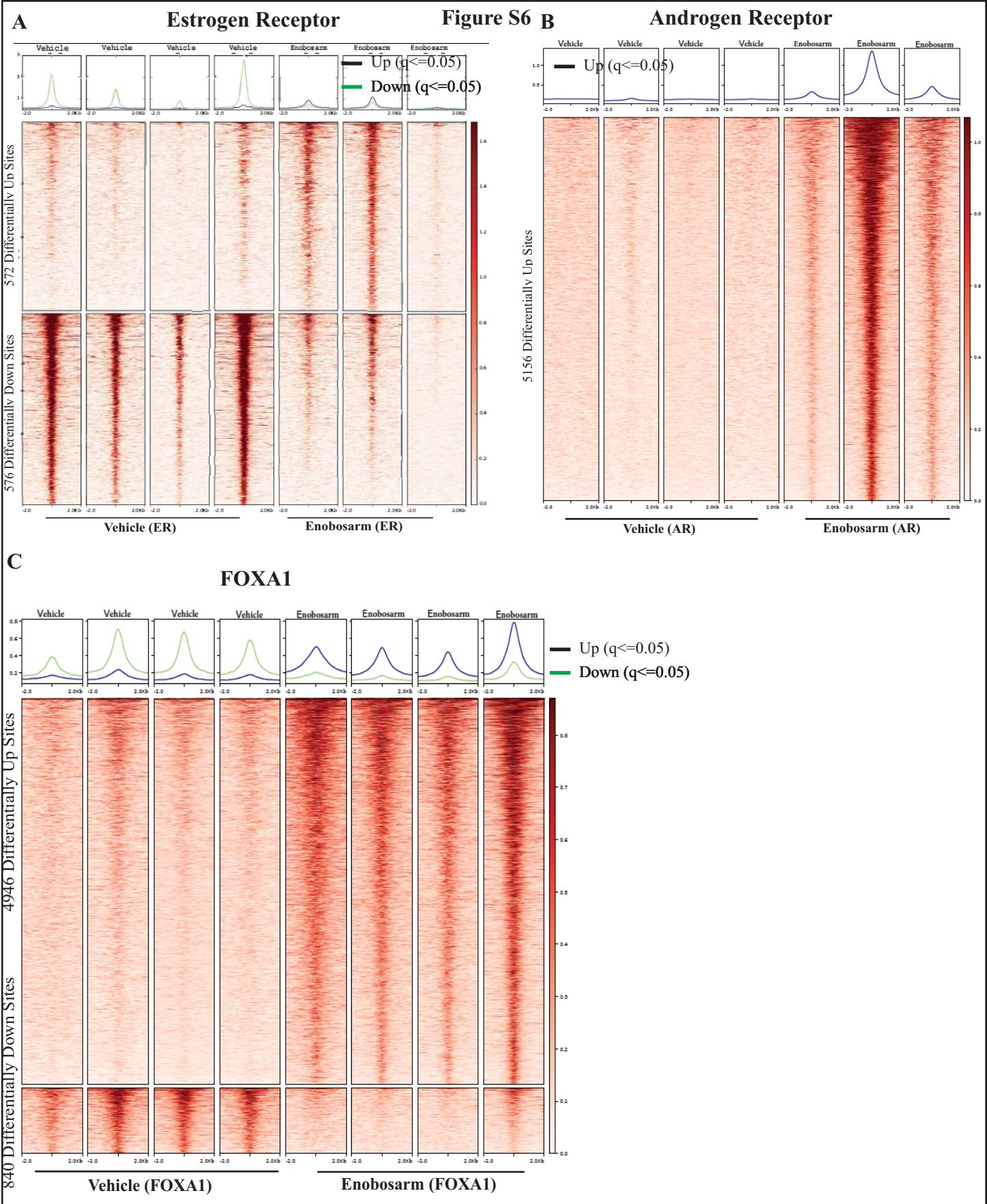



**Figure S6 (Related to Figures 3,4, and 5): Heatmap of ER (A), AR (B), and FOXA1 (C) ChIP-Seq of individual tumor specimens that are represented as averages in figures 3, 4, and 5, respectively**.

# Figure S7

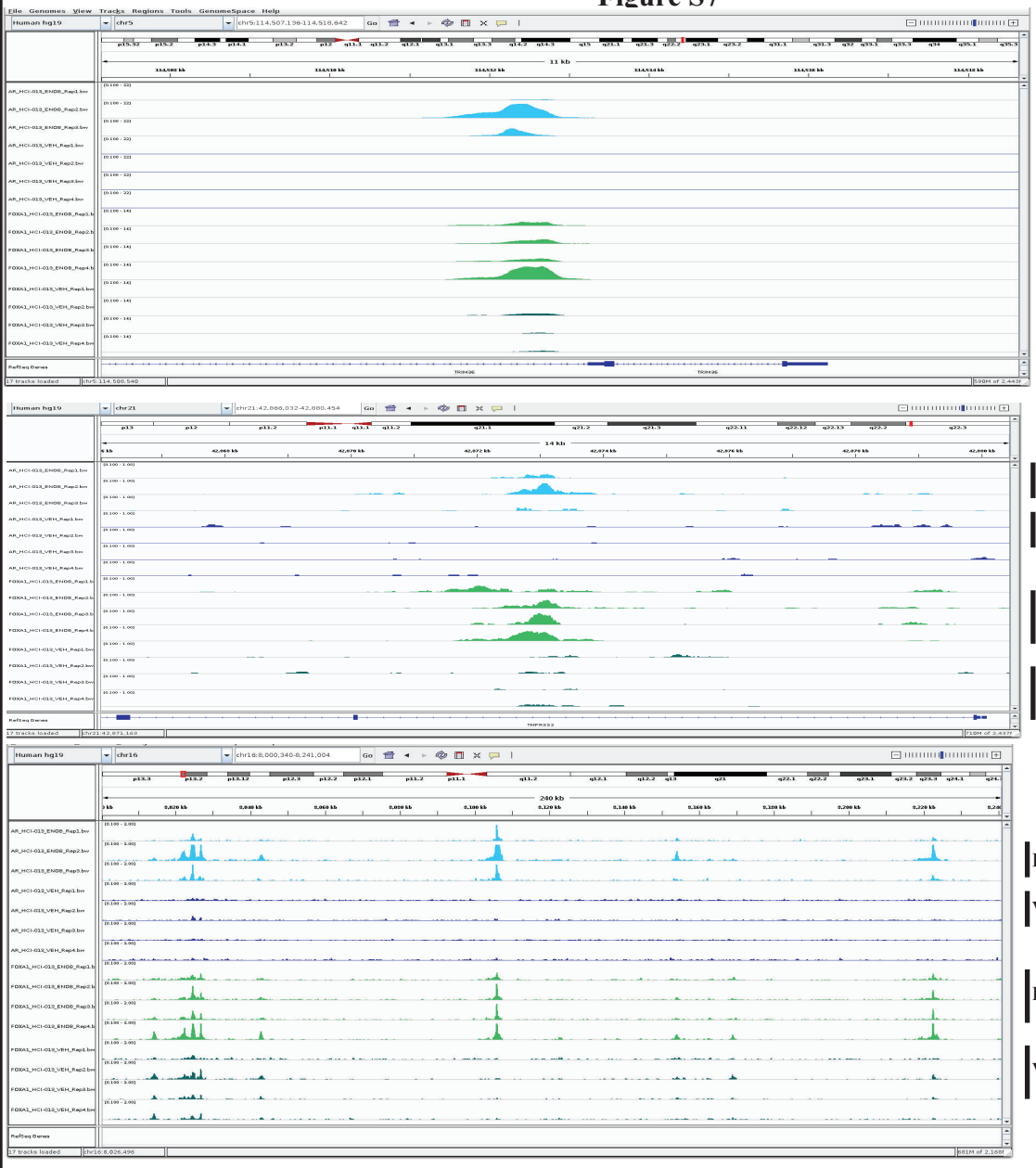
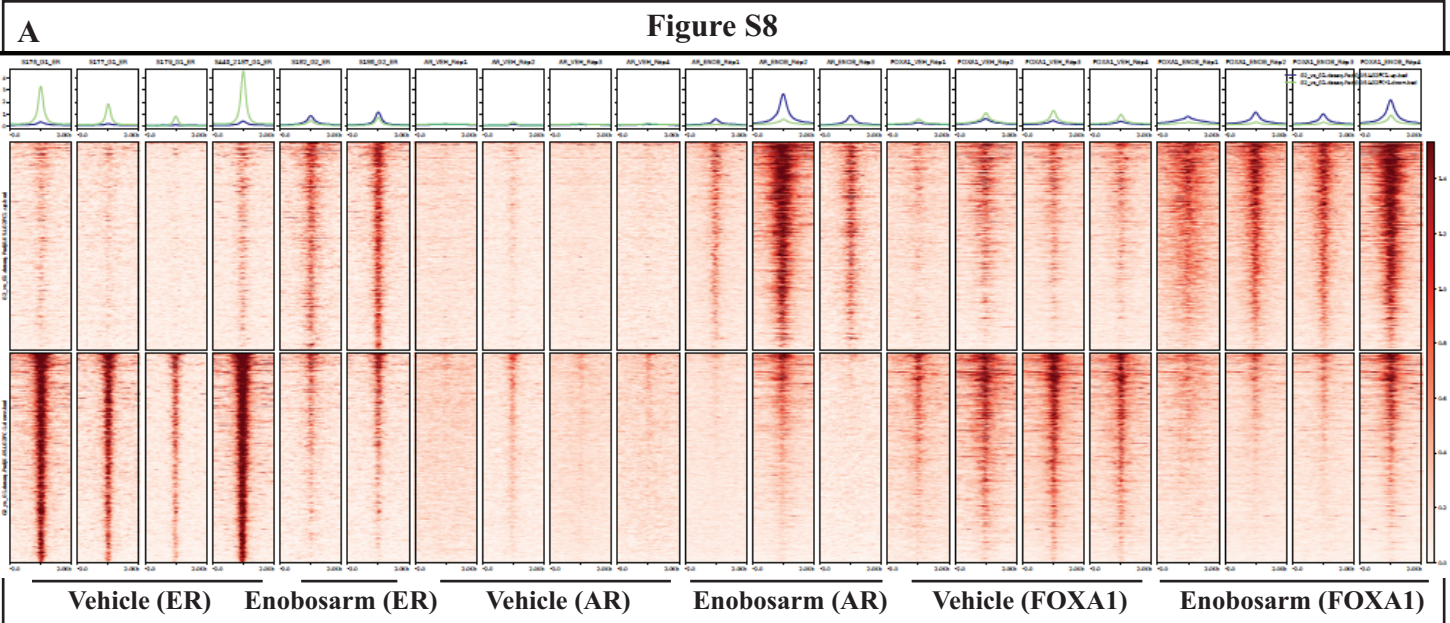



**Figure S7 (Related to Figures 4 and 5): Representative AR and FOXA1 ChIP-Seq peaks.**



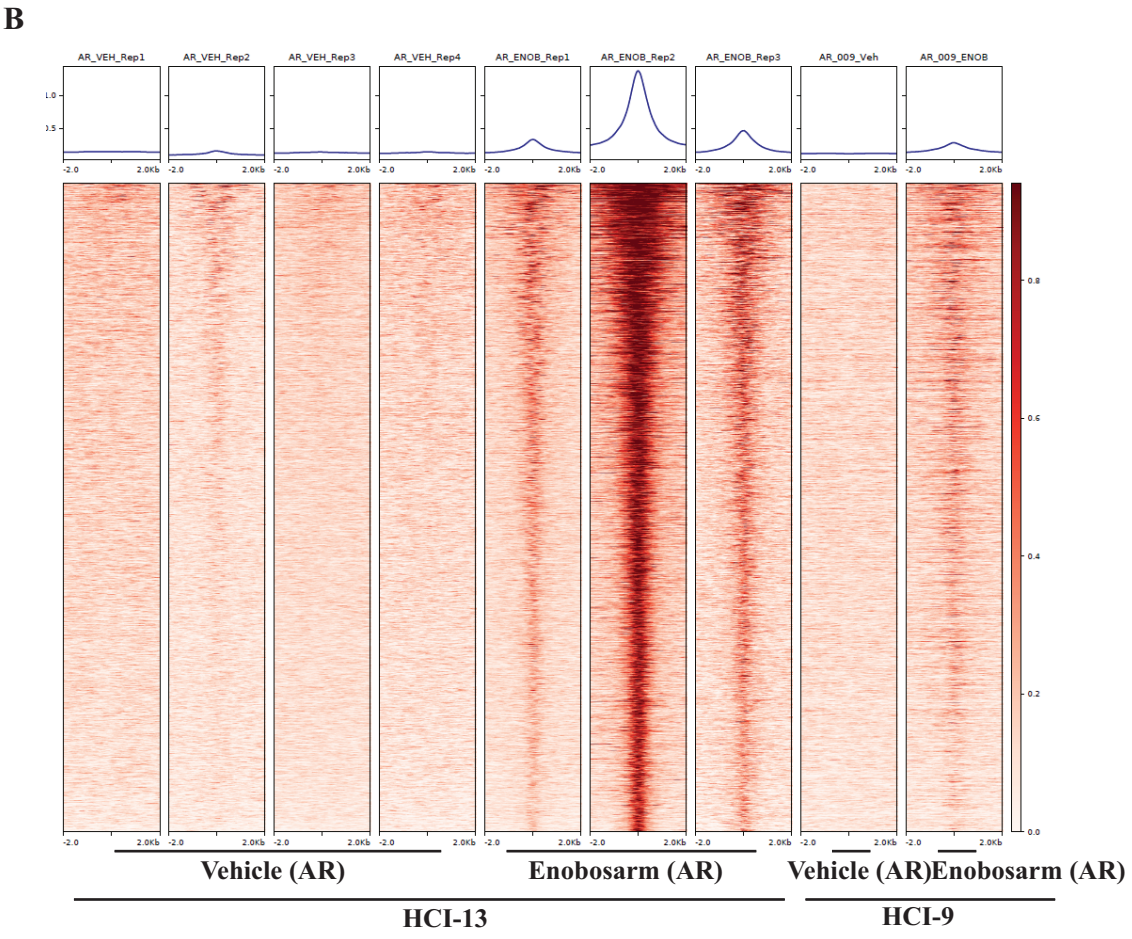




**Figure S8: ER, AR, and FOXA1 DNA binding comparison (Related to Figures 3,4,5, and 7)**. **A**. DNA sequences that were statistically significantly enriched with or depleted of ER in enobosarm-treated samples were aligned to the same regions in AR and ER ChIP-Seq reads and represented as heatmap. **B**. ChIP-Seq for AR in HCI-9 compared to the AR enriched peaks in HCI-13.

**Figure S9**

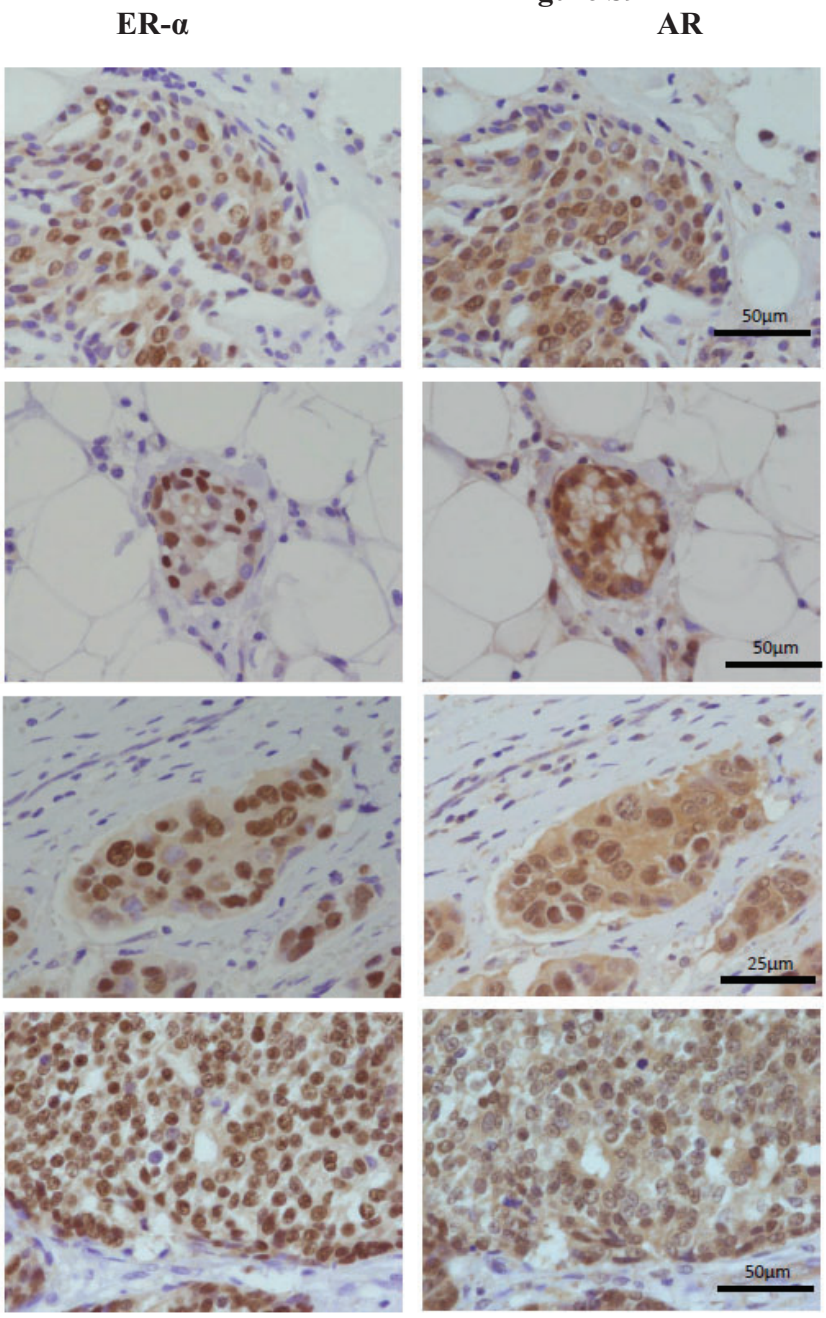



**Figure S9: Colocalization of AR and ER in luminal B breast cancer specimens (Related to Figures 3 and 4).** The scales are provided in each image (25 or 50 µm).

## Figure S10

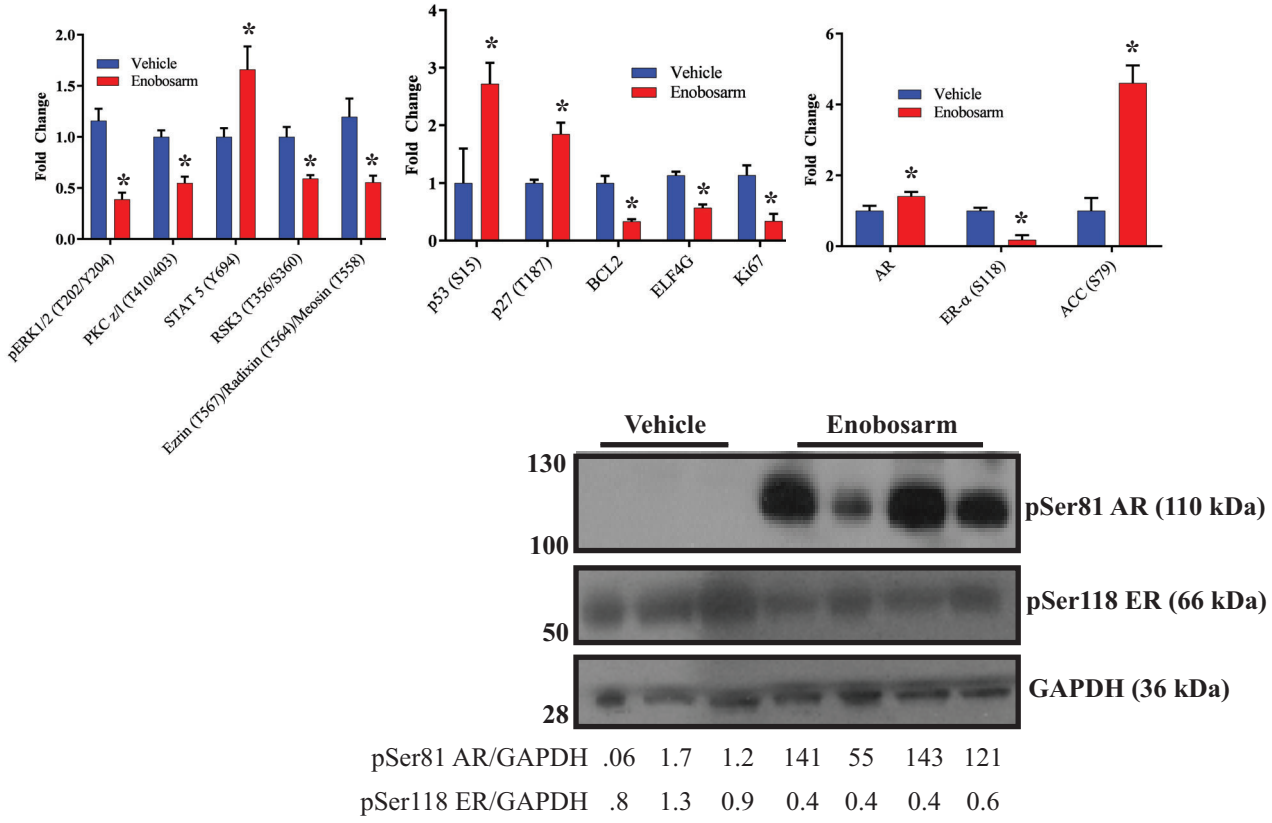



**Figure S10: RPPA-based protein pathway activation analysis of HCI-13 PDX (Related to Figure 1)**. **A**. Lysates from HCI-13 tumor specimens (n=4) from PDX (**Figure 2B**) were printed onto nitrocellulose coated slides. Arrays were probed with a total of 174 antibodies targeting a wide range of protein kinases and their activation via phosphorylation. Arrays were stained with an anti-rabbit or anti-mouse biotinylated secondary antibody. The signals were amplified and a streptavidin-conjugated IRDye680 were used as signal detection methods. Images were acquired and quantified. Western blots were performed in the vehicle and enobosarm- treated samples to validate the results obtained with the RPPA. * $p<0.05$ from vehicle-treated samples; # $p<0.05$ from enobosarm-treated samples. n=3/group (each sample is obtained from 5 individual fragments. PDX-patient-derived xenograft; HCI-Huntsman cancer institute.

## Table ST1

| Patient ID | ER (%) | PR (%) | HER2 (of 3) | Ki-67 (%) | Type | Treatments prior to sample collection |
|---|---|---|---|---|---|---|
| 1005 | 90 | 90 | 1+ | 12 | Adenocarcinoma | No previous treatment |
| 1075 | 30 | 10 | N.D. | 70 | Invasive ductal carcinoma | Neoadjuvant (taxol) |
| 1074 | 90 | N.D. | 0-1+ | 19 | Invasive lobular carcinoma | Radiation, tamoxifen |
| 1073 | 95 | 95 | 1+/3+ | 8 | Infiltrating ductal carcinoma | No previous treatment |
| 1053 | 100 | 0 | 3+ | N.D. | Infiltrating lobular carcinoma | Taxol, Herceptin |
| 1050 | 100 | 84 | 0 | N.D. | Infiltrating ductal carcinoma | No previous treatment |
| 1045 | 100 | 90 | N.D. | N.D. | Infiltrating lobular carcinoma | No previous treatment |
| HCI-13 | + | + | + | | Infiltrating lobular carcinoma. Bone, brain, lung, pericardium, liver mets | Leuprolide, letrozole, exemestane, tamoxifen, zoledronic acid, cyclophosphamide, methotrexate, 5-fluorouracil, paclitaxel, doxorubicin, carboplatin, gemcitabine |
| HCI-7 | + | + | + | N.D. | Luminal B | Paclitaxel, doxorubicin, gemcitabine, carboplatin |
| HCI-9 | - | - | - | N.D. | Poorly differentiated adenocarcinoma | Cyclophosphamide, paclitaxel, 5-fluorouracil, anastrazole, fulvestrant, zolendronic acid |

N.D. Not Done

**Supplementary Table ST1 (Related to Figure 1): Characteristics of patient specimens used in preclinical studies**.