Supplementary Methods

Cell culture conditions. BCK4 and MCF7 cells were cultured in MEM with 5% FBS, NEAA, and insulin. MCF7-TamR cells were cultured in phenol-red free IMEM supplemented with 11.25 nM insulin, 5% CSS, and 100 nM 4-OH-tamoxifen. ZR-75-1 cells were cultured in RPMI with 5% FBS, T47D cells were cultured in DMEM with 10% FBS. PT12 cells were cultured in DMEM/F12 media supplemented with 10% FBS, $1x10^{-9}$ M Insulin, and 100 ng/ml cholera toxin. All cells were cultured in a 37°C incubator with 5% CO_2. Luciferase-expressing MCF7 cells were generated by stable infection with pLNCX2-GFP-Luc vector encoding a GFP and luciferase, and sorted for GFP-expressing cells by flow cytometry.
MCF7 AR knockdown cells were generated by lentiviral transduction of shRNAs targeting AR (pMISSION VSV-G, Sigma Aldrich; St Louis, MO), including AR shRNA 3715 (shAR15) and AR shRNA 3717 (shAR17). Lentiviral transduction of pMISSION shRNA NEG (shNEG) was used as a non-targeting control. Plasmids were purchased from the University of Colorado Functional Genomics Core Facility.

Tumor Xenograft Studies. MCF7 experiments with enzalutamide delivered in rodent chow were performed at the University of Colorado Anschutz Medical Campus and approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC protocol 83611(03)1E). All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. For MCF7 xenograft experiments, $1x10^6$ MCF7-GFP-Luc cells were mixed with growth factor-reduced Matrigel (BD Biosciences) and injected bilaterally into the fourth inguinal mammary fat pad of female, ovariectomized athymic nu/nu mice (Taconic). At time of tumor injection, E2 pellets (60-day release, 1.5 mg/pellet, Innovative Research of America) were implanted subcutaneously (SQ) at the back of the neck. Once the tumors became established, mice were matched into groups by total tumor burden as measured by IVIS or caliper. Groups receiving tamoxifen had a 90-day release, 5 mg/pellet

(Innovative Research of America) implanted SQ. Mice were administered Enza in their chow (approximately a 50 mg/kg daily dose). Enza was mixed with ground mouse chow (Cat # AIN-76, Research Diets Inc; New Brunswick, NJ) at 0.43 mg per gram of chow. Mice in the control group received the same ground mouse chow without Enza. Feed was changed in the animal cages twice a week. Water and feed were prepared ad libitum. Two hours prior to sacrifice, mice were injected IP with 50 mg/kg BrdU (Sigma-Aldrich). Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and blood, tumors, colon, uteri and mammary glands were harvested. The MCF7-TamR xenograft experiment was performed as described above, except mice did not receive implantation of hormone pellet. For the PT-12 xenograft study, 6 x 10^6 cells were injected into the fourth inguinal mammary fat pad of NOD-SCID-IL2Rgc–/– female mice into which a DHT pellet (1.5 mg 60-day release, Innovative Research of America) or E2 pellet (1.5 mg 60-day release) was implanted SQ.

Immunohistochemistry. Antibodies used were: AR clone 441, AR clone SP107 (PT12 xenograft) (Ventana Medical Systems, Tucson, AZ), ER clone 1D5 (clinical samples) (Dakocytomation, Carpinteria CA), ER SP1 (xenografts) (Thermofisher, Grand Island, NY), BrdU #347580 (BD Biosciencies, San Jose, CA), and Cleaved Caspase 3 #9661 (Cell Signaling Technologies, Danvers, MA). Slides were deparaffinized in a series of xylenes and ethanols and antigens were heat retrieved in either 10mM citrate buffer pH 6.0 (BrdU, Cleaved Caspase 3, ER SP1), 10mM Tris/1mM EDTA buffer pH 9.0 (ER 1D5) or BORG pH 9.5 retrieval solution (AR) (Biocare Medical, Concord, CA). Tissue for BrdU was incubated in 2N HCl followed by 0.1M sodium borate following antigen retrieval. Tris buffered saline with Tween (0.05%) was used for all washes. The Vectastain Universal Elite ABC horseradish peroxidase (HRP) kit (Vector Laboratories, Burlingame, CA) was used to detect AR, and all other antibodies were detected with Envision-HRP (Dakocytomation) followed by DAB+ (Dakocytomation) for chromagen visualization.

For BrdU and Caspase 3 staining in xenograft studies, three separate 200X fields of each xenograft tumor were taken using an Olympus BX40 microscope (Center Valley, PA) with a SPOT Insight Mosaic 4.2 camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI). A color threshold (RGB for positive staining nuclei, and HSB for total nuclei) was adjusted manually using ImageJ for each image, and particles created by the thresholds were analyzed for total area. RGB area was divided by HSB area and multiplied by 100 for each image.

Immunofluorescence. $1.5x10^4$ MCF7 cells were plated in 8-well chamber slides and hormone starved in phenol red-free media with 5% CSS for 72hrs. Cells were then pre-treated with Enza or bicalutamide (bic) for 3hrs, then treated with hormones +/- Enza or bic as described for 3 hrs. The cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 30 minutes at room temperature and permeabilized with 0.2% triton X-100. Samples were then blocked with 5% bovine serum albumin for 1 hour and incubated with an antibody against AR (PG-21, EMD Millipore, 1:100) and ER (Ab16, Thermo) in PBS 0.1% triton overnight. Incubation with the secondary antibody anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 680 was performed in 2.5% bovine serum albumin for 2 hours at 4 C followed by additional PBS washes. Cells were visualized with a 20x objective.

EC50 Determination. 1000 cells/well for MCF7 or 2000 cells/well for T47D were plated in a 96-well plate. After 24hrs, culture medium was removed and replaced with 80µl media supplemented with 5% CSS. Different concentrations of Enza were added to each well, followed by adding E2 to a final concentration of 10nM. Test plates were incubated for 8 days in the humidified incubator at 37°C with 5% CO2, and cell survival was determined using the CellTiter-Glo Assay (Promega, Madison, WI). In order to calculate absolute IC50 (EC50), a dose-response curve was fitted using nonlinear regression model with a sigmoidal dose response. The EC50 values were calculated according to the dose-response curve generated by GraphPad Prism 6.0.